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ROLE OF PLEURAL FLUID BIOMARKERS-ADA, ADA2 & INTERFERON GAMMA IN THE DIAGNOSIS OF TUBERCULOUS PLEURAL EFFUSION.

C E R T I F I C A T E

This is to certify that the dissertation entitled "ROLE OF PLEURAL FLUID BIOMARKERS-ADA, ADA2 & INTERFERON GAMMA IN THE DIAGNOSIS OF TUBERCULOUS PLEURAL EFFUSION" is the bonafide original work of **Dr. A.Ashwin Oliver**, towards the M.D. Branch- I (Pulmonary Medicine) Degree Examination of the Tamil Nadu

Dr. M.G.R University, Chennai to be conducted in 2014.

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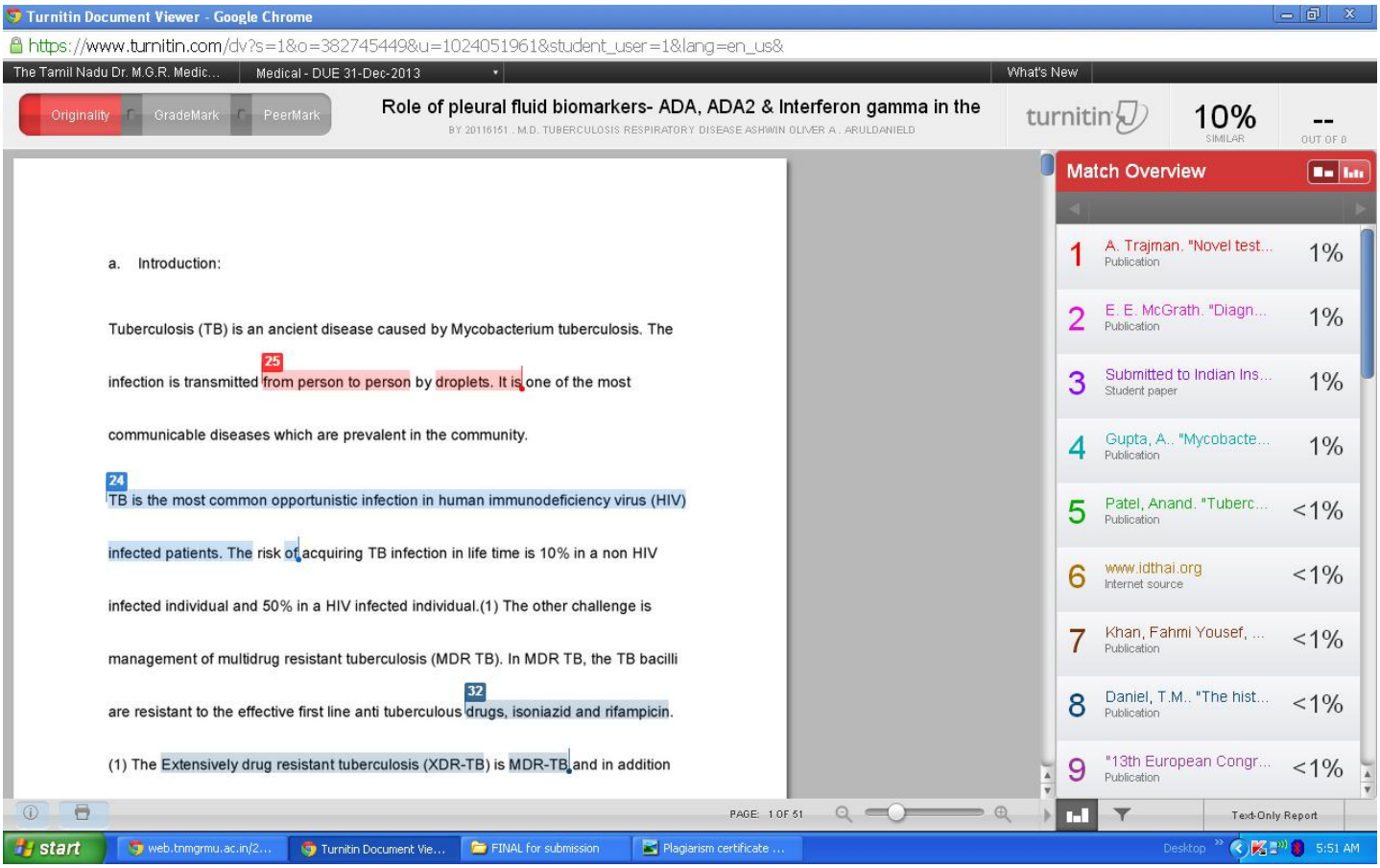
PLAGIARISM CERTIFICATE



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ROLE OF PLEURAL FLUID BIOMARKERS-ADA, ADA2 & INTERFERON GAMMA IN THE DIAGNOSIS OF TUBERCULOUS PLEURAL EFFUSION.

1. AIMS & OBJECTIVES OF THE STUDY:

a) To study the role of pleural fluid biomarkers- ADA, ADA2 and Interferon gamma in the diagnosis of tuberculous pleural effusion.

b) To study the combination of biomarkers in the diagnosis of pleural tuberculosis.

2. REVIEW OF LITERATURE:

a. Introduction:

Tuberculosis (TB) is an ancient disease caused by Mycobacterium tuberculosis. The infection is transmitted from person to person by droplets. It is one of the most communicable diseases which are prevalent in the community.

TB is the most common opportunistic infection in human immunodeficiency virus (HIV) infected patients. The risk of acquiring TB infection in life time is 10% in a non HIV infected individual and 50% in a HIV infected individual.(1) The other challenge is the management of multidrug resistant tuberculosis (MDR TB). In MDR TB, the TB bacilli are resistant to the effective first line anti tuberculous drugs, isoniazid and rifampicin. (1) The Extensively drug resistant tuberculosis (XDR-TB) is MDR-TB and in addition resistant to fluroquinolones and Injectables.

b. History of tuberculosis:

Tuberculosis is considered one of the ancient diseases in the pre-historic era. It was an epidemic in the eighteenth and nineteenth century gaining the name "Captain among these men of death" (2). Tuberculosis caused the "white plague" in seventeenth and eighteenth century in Europe(3). In 1882, Dr.Robert Koch discovered the tubercle bacilli. The administration of tuberculin protein and demonstrating tuberculin sensitivity was started by Clemens Freiherr von Pirquuet in 1907. (2) The practice of open air treatment and sanatorium was started in 1907.In 1921, the first BCG vaccine was administered to aninfant bornof a mother dying of pulmonary tuberculosis and the infant survived.A mass BCG vaccination was conducted in Europe by the UNICEF and Danish Red Cross. This was the first disease control program conducted by an agency of the world health organization. (2)

The first antituberculous drugs Para-amino salicylic acid (PAS) and Thiosemicarbazone were discovered in the 1940's but had disappointing results due to the bacteriostatic nature of the drugs. Streptomycin was discovered in 1944 was used to treat TB and showed promising results. Then newer drugs like Isoniazid(INH) and Rifamycin's were discovered in 1952 and 1957 respectively.(2) The discovery of newer effective chemotherapeutic drugs led to the treatment of TB in domiciliary basis.

c. Microbiology of Mycobacterium tuberculosis:

Tuberculosis is caused by Mycobacterium tuberculosis (MTB). It is also called as Koch's bacilli(4). It belongs to the genus Mycobacterium and the most important member of the genus is Mycobacterium tuberculosis. The microbiological nature of Mycobacterium tuberculosis is its aerobic, non motile, non spore forming bacillus. It has a cell wall with high lipid content which accounts for the impermeability and resistance to antimicrobial

agents. (5) The MTB are grown in Middlebrook's medium which is an agar based medium and Lowenstein-Jensen's medium which is an egg based medium. These organisms cannot be stained by the routine gram stain. These organisms are acid fast and are stained using the Zeil-Neelsen stain using carbol fuchsin(3)

d. Pathogenesis:

The MTB targets the host macrophages, which are the key mediators for innate and adaptive immune response. The organism gains access into the macrophage by the process of receptor mediated phagocytosis. The ability of the macrophages to produce anti-microbial chemicals like nitric oxide and reactive oxygen intermediates is blunted.(5)

The components of the MTB are recognized by multiple pattern recognition receptors on the alveolar macrophages which initiates innate immunity. The circulating monocytes migrate across the blood vessels to the site of infection in response to the chemokines. Within the tissues, the monocytes differentiate into macrophages

and help in the process of ingestion & killing of the organism.(5) The macrophages are activated by interferon gamma which is secreted by T cells. The interaction between the macrophages and T cells is the central in elimination of the organism.

Adaptive immunity which is regulated by the CD4 and CD8 cells arrest the bacterial growth and lead to latent TB infection. It is a state of dynamic bacterial and immunological equilibrium and not merely a state of bacterial stasis(5)

The proposed mechanisms for reactivation of latent TB infection are qualitative and quantitative defects of CD4 cells and therapeutic neutralization of tumour necrosis factor(5).

e. Diagnostic methods for tuberculosis:

Sputum microscopy:

Sputum microscopy and Ziehl Neelsen staining for acid fast bacilli is done to any person who has prolonged duration of cough for 2 weeks or more, with or without symptoms of Tuberculosis. Two

sputum specimens collected on consecutive days. The first sample is a spot sample and second being an early morning sample. A patient who is suspected to have extra-pulmonary tuberculosis should also undergo sputum examination if he has cough of any duration. The sensitivity of sputum microscopy is 64% and a specificity of 98%(6). The advantage of sputum microscopy is its simple, rapid and inexpensive. The sensitivity of the test decreases if the bacterial load is less than 10,000 organisms per mL sputum sample. (7) The paediatric population, HIV co-infected patients and in extra pulmonary tuberculosis patients are the other factors which affect the sensitivity of sputum microscopy.

Fluorescent microscopy:

In Fluorescent microscopy, flurochrome dyes are used to stain. It consists of a halogen or high pressure mercury lamp which is the light source and is used to excite the dye and florescence. Fluorescent microscopy has a higher sensitivity than conventional

sputum microscopy by 10%.The higher cost(7) is the major limiting factor in fluorescent microscopy.

Culture methods:

The culture of Mycobacterium tuberculosis is the gold standard for the diagnosis of tuberculosis. There are two types of culture media: 1) Solid medium, 2) Liquid medium.

The solid medium culture is the Lowenstein-Jensen medium and Middle Brook medium. The liquid mediums are the liquid culture medium methods with automated incubation and reading systems. They are MB/BacT ALERT 3D system and BACTEC MGIT 960 system.

Lowenstein-Jensenmedium (LJ medium):

The LJ mediumis a solid media used for growing Mycobacterium tuberculosis and also used for drug susceptibility testing. Its main contents are fresh egg and glycerol. The two advantages of egg

based media are: 1) It can support the growth of wide variety of Mycobacterial species; 2) Niacin testing can be done on egg media. (8) The initial Lowenstein media was modified by Jensen by removing Congo red, increasing the concentration of malachite green and alternating the citrate & phosphate contents. The median duration of growth to appear in LJ media is about 3 to 6 weeks for isolation and 1 to 2 weeks for speciation(9). The major disadvantage is the prolonged turn around time for the diagnosis.

Middle Brook medium:

It is another solid medium which is used for Mycobacterium culture. It uses agar as a primary substrate. The major drawback of this medium is the long period for growth, at least 3-4 weeks.(10)

BACTEC 460:

This liquid media was found by Deland and Wagner in 1969. It is a radiometric technique using ^{14}C- substrate incorporated in the growth media. As the bacterium grows, it utilizes the ^{14}C thereby releasing ^{14} carbon dioxide which is thereby identified by

radiometric technique. It was found that this method had a 15 to 20% increased culture positivity compared with the conventional solid culture media. (10) The limitation with liquid media is risk of contamination. Addition ofantimicrobials to the media prevents contamination and it was called BACTEC 460TB. The antimicrobials added in the culture media are Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin and the combination is called PANTA formulation. The major disadvantage with BACTEC 460TB media is the use of radioactive substrate, ^{14}C. Strict handling and disposal measures of radioactive substances(10) is required while using this method.

Mycobacterium growth indicator tube (MGIT):

The MGIT culture method is a non- radiometric technique developed by Becton-Dickson and company. It is a liquid broth medium with 7mL of modified Middlebrook 7H9 broth base. The medium is further enriched with Oleic acid, albumin, dextrose and catalase to form MGIT OADC or MGIT 960 growth supplement to

make the medium complete. The sterilization of the medium isdone by autoclaving.(10) Except blood, all the samples can be processed using MGIT culture method. The MGIT tube contains oxygen quenched flurochrome, tris 4, 7-diphenyl-1, phenonthroline ruthenium chloride pentahydrate embedded in silicone at the bottom of the tube. With the bacterial growth, the oxygen is utilized and carbon dioxide is released. With depletion of oxygen, the flurochrome is not inhibited resulting in fluorescence when visualized under ultraviolet rays. The growth is visualized as non-homogenous turbidity or small granular or flaky appearance. The test is considered negative if there is no growth at 6 weeks (42 days) and it is considered negative(10). In a study, 9558 extra pulmonarysamples were cultured with BACTEC MGIT 960 tube and LJ cultures; the sensitivity was 88.8% and 69.3% respectively(11). The advantages of MGIT: 1) It does not use radioactive substance, 2) There is rapid growth compared to LJ and Middlebrook media, 3) The growth can be visualized.

Molecular diagnostic methods:

Line probe assay (LPA):

In the era of increasing drug resistant TB, the rapid diagnosis and initiation of appropriate chemotherapeutic drugs is the key for successful treatment. The conventional culture and drug sensitivity methods take 2 to 8 weeks and additional 3 to 6 weeks respectively. This led to the emergence of newer diagnostic methods using molecular probes and line probe assay is the first of its kind. The line probe assay is a reverse hybridization based assay which is used for rapid detection of mutation for INH and Rifampicin. The advantage of LPAs is it has a high sensitivity (90-97%) and specificity (≥99%) in detecting resistance to Rifampicin and or INH(12). The LPA is done in specialized lab with following areas: 1) pre-amplification area (reagent preparation), 2) DNA extraction area(specimen preparation) , 3) amplification area(template addition and amplification), 4) Post amplification or hybridization/ detection area. The molecular

identification of resistance is detected by identifying 81-base pair region mutation of rpoB gene and katG gene mutation for Rifampicin and INH respectively. The major steps in LPA are 1) NaOH-NALC decontaminated sputum or culture isolates for DNA extraction, 2) multiplex PCR on the extracted sample, 3) Reverse hybridization where the resistance to Rifampicin and or INH is detected on strips. There are newer line probe assays looking at gyrA mutations for fluroquinolone resistance(13)From 2010, The line probe assay is included by the RNTCP programmefor the diagnosis of MDR tuberculosis (14)

Xpert MTB/Rif:

Thisis an automated molecular test for Mycobacterium tuberculosis and to detect resistance to Rifampicin. The principle of the test is hemi-nested real time polymerized chain reaction (PCR) and also amplifies MTB sequence of rpoB gene which reflects resistance to Rifampicin(15)

The Gene Xpert test consists of a plastic catridge which contains reagents required for bacterial lysis, nucleic acid extraction, amplication and amplicon detection. The initial step is addition of bactericidal buffer to sputum sample followed by adding the sputum to catridge. The results are obtained after 2 hours. The advantage of this test is quick diagnosis with identifying Rifampicin resistance (16)The disadvantages are short half life (18 months), requires stable electrical supply, annual recalibration, ceiling temperature, higher cost.

Mantoux test/ Tuberculin skin test:

The tuberculin skin testing is one of the tests used to detect tuberculosis infection. It also assesses the status of infection in community. The PPD-S is currently used. The Tuberculin(PPD-RT 23 with Tween 23) at a dose of 5 tuberculin units is administered intradermally and the reading is taken after 48 to 72 hours. The preferred site of administration is the volar aspect of left forearm. The immunological basis for Mantoux test is delayed

hypersensitivity reaction. In sensitized individuals, the T cells release lymphokines which cause induration.

Table 1 showing interpretation of Mantoux test(17)

5mm or more	10mm or more	15mm or more
Recent contact with TB case	Injectable drug abusers	Persons with no risk factors for TB
HIV positive person	Mycobacteriology lab personnel	
Organ transplant recipients	High risk clinical conditions like diabetes	
Long term corticosteroids	Infants, children exposed to adults in high risk categories	
	Recent arrivals < 5 years from high prevalence country	

Table 2 showing false positive and false negative for Mantoux test(17):

False positive	**False negative**
Atypical mycobacterial infection	Recent TB infection
BCG vaccination in the past	Very old TB infection.
Wrong method of administration of TST	Very young age
Wrong interpretation	Recent vaccination- Measles
Wrong antigen	Wrong administration and interpretation
	Cutaneous anergy

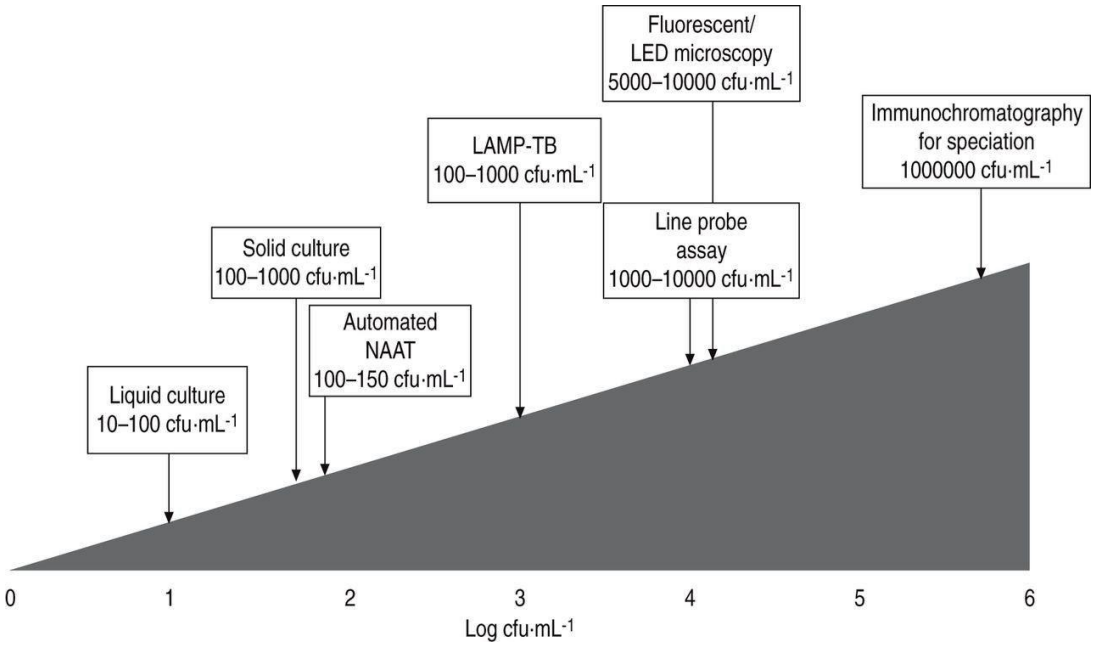

Figure1 showing sensitivity of the current diagnostic tests(5)

Most sensitive tool being automated liquid cultures.

Automated nucleic acid amplification technique (NAAT) shows a compared sensitivity to solid cultures.

LAMP- Loop mediated amplification.

TB- Tuberculosis; LED- Light emitting diode.

f. Incidence of Tuberculosis:

Global incidence:

The global incidence of tuberculosis in 2011 was 8.7 million(18)

India:

The incidence in 2011 was 2.2 million and the prevalence was 3.1 million.(18)The WHO data reveals that 6,88,530 patients were detected to have TB and HIV co-infection in 2011 and 2.1% of the newly detected cases to be MDR TB(19)

Tamilnadu:

The total population covered by RNTCP was 72,100,000 and 79,830 patients registered under RNTCP for treatment in the year 2011(18)

g. Tuberculous pleural effusion:

TB pleural effusion is the commonest cause of pleural effusion inmany countries including India. TB pleural effusion is the common site for extrapulmonary tuberculosis(20). Extrapulmonary tuberculosis has increased in prevalence to four fold with the increase in HIV infection.

h. Incidence of TB pleural effusion:

Tuberculous pleural effusion varies in incidence from country to country. The incidence in United States is 3-5% whereas its 25% in Burundi, an East African countryand 20% in South Africa(21). The patients with immunocompromised state are more prone to develop TB pleural effusion. The incidence of pleural tuberculosis is comparatively high in India and it accounts about 30%(22).

i. Pathogenesis of TB pleural effusion:

TB effusion is described as an acute granulomatous pleuritis(23). It is a sequlae of a recent tuberculous infection within 6 to 12 weeks. In older individuals, it occurs as classical reactivation tuberculosis. There may be no apparent evidence of tuberculosis radiographically.

The pathophysiology of TB pleural effusion is believed to be rupture of subpleural caseous focus in the lung into the pleura.

The other mechanism believed is delayed hypersensitivity reaction(21).When tuberculous protein gains access to pleural space the delayed hypersensitivity reaction takes place. The evidence for delayed hypersensitivity is demonstrated by intrapleural administration of tuberculin protein in purified protein derivative (PPD) sensitized guinea pigs lead to development of exudative pleural effusion(21). Anti lymphocyte serum in sensitized guinea pigs suppresses the development of pleural effusion. The mycobacterial cultures in the pleural fluid are

negative in most of the patients supporting delayed hypersensitivity reaction.

Once the delayed hypersensitivity reaction occurs, the permeability of the pleural capillaries increases leading to excessive movement of protein into the pleural space and the volume of pleural effusion increases. In addition the clearance of pleural fluid is affected by lymphatic obstruction due to lymphocytic pleuritis(21).

j. Characteristics of TB pleural effusion:

The aetiology of TB pleural effusion is inflammation and is invariably an exudate. The cellular composition of the pleural fluid is predominantly lymphocytic. Tuberculous pleural effusion rarely presents with pleural fluid eosinophilia (Eosinophil count >10cells) but can occur in pneumothorax or with previous thoracocentesis(21)

The effusion can be neutrophilic in a setting of tuberculous empyema(24). The pleural fluid glucose may be reduced or at the

serum level. The pleural fluid pH is around 7.30 and LDH is elevated above serum levels.

According to an Indian study(23)which looked at the clinico-radiological and biochemical characteristics of TB pleural effusionin 94.34%,pleural fluid protein was >3gm/dL, in 97.44% pleural fluid LDH was >200IU/L and in 83.02% pleural fluid glucose was <60mg/dL.

k. Clinical features of TB pleural effusion:

TB pleural effusion is commonly seen in middle age to older age group. The onset of illness is abrupt in two-third of the patients and insidious in rest of the cases. The common symptoms are non-productive cough (~70%), pleuritic chest pain (~70%)(21). Most of the cases have febrile, approximately 15% are afebrile. The pleural effusion in usually unilateral and most commonly involves one half of the hemithorax(25). After malignancy and pneumonia, tuberculosis is the third most common cause for large pleural effusion. About 20% of patients have co-existing parenchymal

lesions on chest x ray & 80% of patients have parenchymal lesions on computed tomography(21).

l. TB effusion diagnosis:

The conventional tests used for the diagnosis of pleural tuberculosis are pleural fluid microscopy to look for acid fast bacilli (AFB), pleural fluid MTB culture, pleural tissue MTB culture and histopathological examination of the pleural tissue demonstrating granulomatous inflammation(26). The yield of pleural fluid microscopy in detecting TB pleural effusion is < 5% due to the paucibacillary state. The sensitivity of pleural fluid cultures is low (24-58%) and further limited by the lengthy delay in obtaining results (8weeks). The pleural tissue histopathology and cultures is the currently available sensitive tool to diagnose tuberculous pleural effusion.In addition, pleural biopsy is invasive and the yield depends on the skill of the operator with risk of complications(26)

In a study where they looked at the yield of induced sputum in TB pleural effusion, the sensitivity of induced sputum was 52% when compared with 12% for pleural fluid culture(26)

m. Pleural fluid biomarkers:

Due to thediagnostic challenges in the diagnosis of tuberculous pleural effusion, newer tests and biomarkers are employed to improve thediagnostic yield.

The newer tests are classified as(26):

1) Non specific inflammatory and immune response markers

(E.g. ADA, cytokines, neopterin, leptin, complement activation, Lysozyme)

2) Specific markers to immune response

(E.g. T-cell response to specific antigens- TB SPOT,

Quantiferon TB Gold;B-cell response – antibody detection.

3) Detection of MTB nucleic acid sequences by amplification tests.

4) Scoring system based on combination of markers.

Adenosine deaminase (ADA):

ADA is a non-specific marker of inflammation. It is produced and released from macrophages, activated lymphocytes and neutrophils. ADA1 and ADA2 are the two isoenzymes of ADA. ADA2 is released by activated monocytes and macrophages(27).ADA2 predominantly contributes to the total ADA activity. The accuracy of the test can be improved by analyzing isoenzymes. ADA has a sensitivity of 90.8% and specificity of 82.8%(28)

A study looked at ADA and ADA1/ADA ratio which found a higher sensitivity and specificity when combining ADA ≥40U/L and ADA1/ADA ≤0.42 (29).

	ADA	ADA1/ADAp
Sensitivity	88%	100%
Specificity	92%	98.6%
Positive predictive value	80%	96.4%
Negative predictive value	95.7%	100%
Accuracy	91.2%	99.02%

Table 3: showing the utility of ADA and ADA1/ADAp in the published literature.

Neopterin:

Neopterin is produced by activated macrophages and it's a marker of Th1 activation. Neopterin levels are found high in tuberculous pleural effusion. The sensitivity is 44% and specificity is 85%(26)

Leptin:

Leptin is produced by obese (ob) gene. Low leptin levels are found in active tuberculosis and cancer. But it has a low sensitivity and specificity compared to ADA(26)

Interferon gamma:

The tuberculous infection initiates a cascade of immunological reaction and causes Th1 lymphocyte recruitment and release of various cytokines(30) Among the various cytokines released Interferon gamma is the important cytokine. Among the other cytokines like tumour necrosis factor alpha, IL-1β but the cytokine with high sensitivity and specificity for tuberculosis is interferon gamma. It has a sensitivity of 89% and specificity of 97%.

In malignant effusions higher levels of other interleukins like IL-8, IL-6 and soluble IL-6 receptor and not in TB effusions.(26)

Interferon gamma release assay(IGRA):

The interferon gamma release assays are useful in detecting latent Tb infection. Quantiferon TB Gold and T-SPOT TB test are the commercially available IGRA's. The assay detects the release of Interferon gamma from the mononuclear cells in response to Mycobacterium tuberculosis specific antigen. The T-SPOT TB test carries a sensitivity of 95% and specificity of 76% in pleural fluid samples(26). The Quantiferon TB Gold test uses the ELISA platform and is more amenable to high input and flexibility. Advantage of IGRA is it can detect latent TB infection. Disadvantage of IGRA: 1) Lacks specificity, 2) Costly.

Scoring system based on combination of markers:

A combination of clinical data and biomarkers are used and also found to increase the sensitivity and specificity. A study by Lesley et al found that a combination of ADA> 50 IU/L and lymphocyte neutrophil ratio of 0.75 or more increases the sensitivity and specificity to 88% and 95% respectively(25). A combination of duration of symptoms + protein+ leukocyte count+ lymphocyte %+ ADA increased the sensitivity(31). When ADA>40IU/L combined with Interferon gamma ≥75pg/mL the specificity increased to 100%(32). When ADA>40IU/L combined with ADA1/ADA ratio ≤0.42, the sensitivity increased to 100% and specificity to 98.6%. (29)So by combining different parameters the sensitivity and specificity in diagnosing pleural tuberculosis.

3. METHODOLOGY:

Inclusion criteria:

All patients with exudative pleural effusion getting evaluated in Christian medical college who are willing to participate (after informed consent) were included between the time periods of May 2012- July 2013.

Exclusion criteria:

Patients with proven or suspected malignancy based on imaging or outside biopsy.

Patients not willing to give consent.

Method of recruitment:

Patients who present with pleural effusion to the department of pulmonary medicine, Christian medical college, Vellore were included in the study after obtaining an informed consent. A total of 154 patients were included in the study after obtaining consent.

Method of evaluation:

After recruitment, all patients underwent pleural fluid aspiration followed by pleural biopsy (closed or ultrasound guided).

The pleural fluid samples were sent for cell counts, protein, and LDH and mycobacterial cultures. Using the true cut biopsy needle, pleural biopsy samples were obtained and the samples were sent for mycobacterial culture (LJ medium or MGIT) and histopathology.

A 5mL pleural fluid study sample was collected in a red tube and sent to the clinical biochemistry laboratory.

The sample is then stored at -70degree Celsius. The study samples are stored in deep freezer and pooled followed by analysis.

Adenosine deaminase (Total ADA):

Using the DIAZYME kit, ADA assay was performed; catalog number DZ117A-K and universal configuration.

The principle of the assay is on enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP)(33). The results of ADA are represented in U/L.

Adenosine + H2O $\xrightarrow{\text{ADA}}$ Inosine + NH3

Inosine + Pi $\xrightarrow{\text{PNP}}$ Hypoxanthine+

Ribose-1-phosphate

Hypoxanthine+ 2H2O+ O2 $\xrightarrow{\text{XOD}}$ Uric acid +

2H2O2

H2O2+ 4-AA+ EHSPT $\xrightarrow{\text{POD}}$ 2H2O+ Quinone

Dye

(λ max 556nm)

Adenosine deaminase 1(ADA1):

Colorimetric method was used to analyze ADA1. The substrate adenosine which is used in the estimation of ADA is replaced by the substrate 2'-deoxyadenosine(34).

Adenosine deaminase 2 (ADA2):

The ADA2 activity is calculated from ADA and 2'-deoxyadenosine deaminase activities (34).

Interferon gamma:

The Interferon gamma assay is done using ELISA method. The Interferon gamma assay is done using IFN-γ- EASIA kit with Proprietary name: DIAsource IFN-γ- EASIA kit, catalogue number: KAP1231: 96 tests. It is a solid phase amplified immunoassay performed on microtitre plates. The test uses monoclonal antibodies against epitopes of interferon gamma (35).

The pleural fluid sample of 50µL is pipetted into the appropriate wells followed by adding 50µL of anti-IFN- γ-HRP conjugate into the wells. The kit is allowed to incubate at room temperature for 2 hours at 700rpm±100rpm on a horizontal shaker. The contents in the well are aspirated after incubation followed by washing the plate thrice by 0.4ml wash solution into each well. This step is followed by adding 200µL of freshly prepared Revelation solution into each well and incubation for 15 minutes at room temperature on a horizontal shaker at 700rpm±100rpm. After 15 minutes, 50µL stop solution is added into each well. The absorbencies between

450nm and 490nm are read within 3 hours and the results are calculated.

Sample size calculation:

Data collected from the patients attending the pulmonary medicine,where the investigator is posted.

Sampling strategy:

Sample size is calculated from the formula 4pq/d2. From previous studies, sensitivity and specificity (p) was found to be 90% and precision of the estimate (d) was kept at ± 8 for the calculation of 95% confidence interval (CI). With these values, we calculated a sample size of 80 patients with TB effusions. It is hoped that around 200 subjects with exudative pleural effusion will need to be recruited to obtain this sample size.

4. Data analysis:

The data was analyzed for demographic details, the biochemical parameters and the pleural fluid parameters- cell counts, protein, lactate dehydrogenase (LDH), microbiological variables like pleural fluid culture and pleural biopsy culture. The pleural fluid biomarkers- adenosine deaminase (ADA), ADA1, ADA2, Interferon gamma) are analyzed separately to look for role in diagnosing pleural tuberculosis.

Statistical analysis:

The frequencies and percentages are calculated for the biomarkers- ADA, ADA2 and interferon gamma. The mean age and standard deviation of age is calculated. The sensitivity, specificity, PPV, NPV analysis of ADA, ADA2, and Interferon gamma is done using ROC curve analysis. The area under the curve is also estimated. The mean ADA, ADA2, Interferon gamma was calculated.

5. RESULTS:

The total number of 154 patients was included in the study during the period of May 2012- July 2013. The patients with outside diagnosis of malignancy or radiological evidence of malignancy were excluded. The enrolled patients underwent pleural aspiration and pleural biopsy and the study pleural fluid sample was analyzed for biomarkers- ADA, ADA1, ADA2 and interferon gamma.

Table 4: showing the demographic details of patients with pleural effusion.

Patient characteristics	Total patients= 154
Age, years	44.8
Male	114(74.03))
Female	40(25.97)
Unilateral	146(94)
Bilateral	8(6)
HIV infection	1 (0.64)
Sputum positive for TB	2(1.2)

Demographic details (Table 4):

Among the 154 patients with pleural effusion, male were 74.03% (n=114) and female were 25.97% (n=40%). The mean age at presentation was 44.8 years. The clinical presentation was mostly unilateral pleural effusion, 94%. One patient had HIV infection and two patients had concomitant pulmonary tuberculosis based on sputum examination.

Age distribution of diseases: (Fig 2)

Figure 2 shows the age distribution among the study patients. The mean age group of patients with tuberculous pleural effusion is 39 years. The older patients with pleural effusion were often diagnosed to have neoplastic etiology. The mean age for malignancy was 53 years and for lymphoma 47 years.

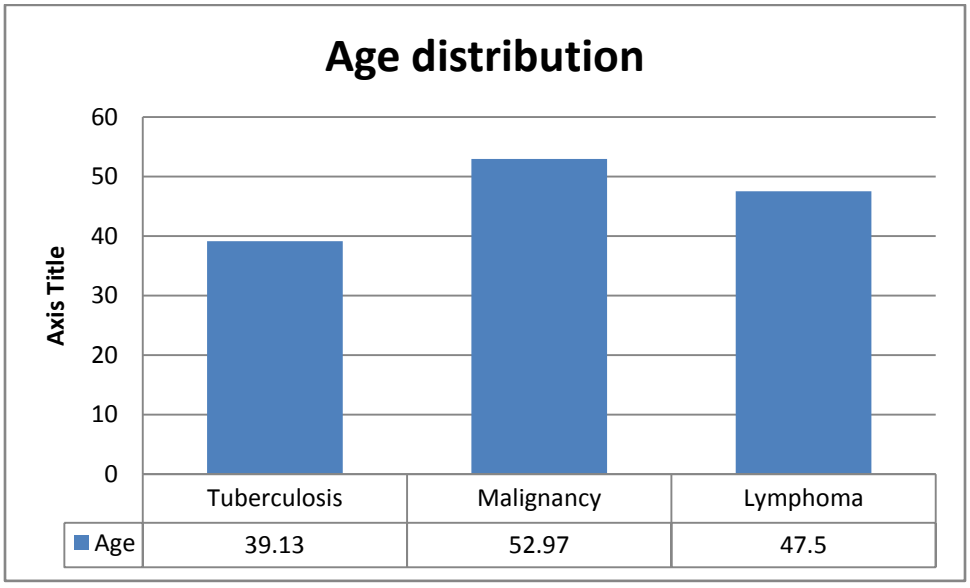

Fig 2: showing age distribution in TB, malignancy & lymphoma.

Gender distribution in TB pleural effusion (Fig 3)

Among the 68 patients with tuberculous pleural effusion, 53 were male (78 %) and 15 were female (22%), with a male: female ratio of 3.5: 1.

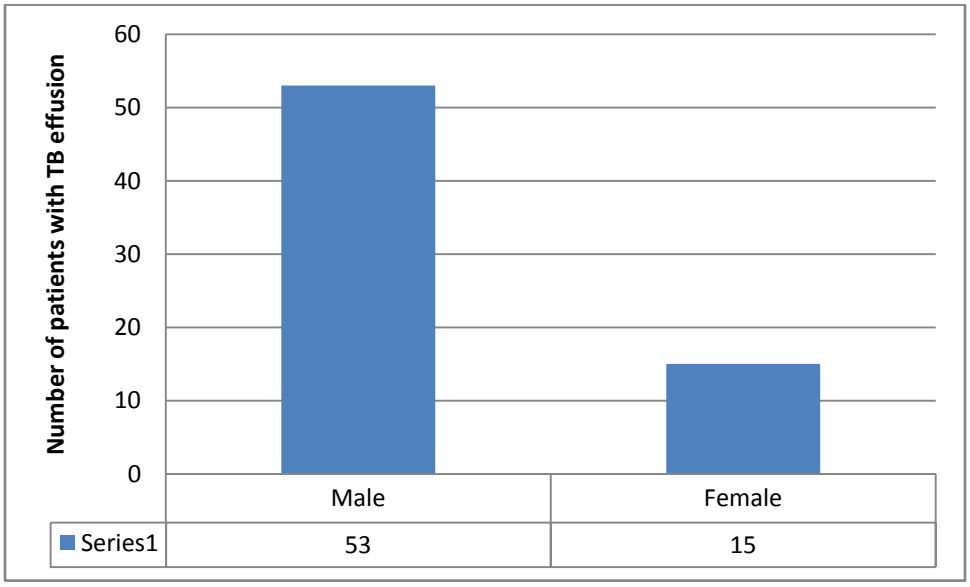

Fig 3: shows the gender distribution of TB pleural effusion.

Pleural fluid cellular characteristics (Table 5)

The pleural fluid characteristics were analyzed and all patients had exudative pleural effusion based on Light's criteria.

80% of the patients had lymphocytic exudative pleural effusion and the predominant underlying disorders in these were tuberculosis and malignancy.

24 patients had neutrophil predominant effusion and the aetiology in them was para-pneumonic effusions. A small subset of about 6 patients had eosinophilic effusion.

Cell counts	Number of patients & percentages N (%)
Lymphocyte	124 (80)
Neutrophil	24 (15.58)
Eosinophil	6 (3.89)

Table 5: showing the cellular characteristics in pleural fluid

Pleural fluid biochemical parameters (Fig 4 &5)

The pleural fluid biochemical parameters were analyzed with the etiology. Tuberculosis had the highest mean pleural fluid protein content, followed by malignancy and lymphoma. The pleural fluid lactate dehydrogenase (LDH) showed highest levels in malignancy, followed by lymphoma and Tuberculosis had the least.

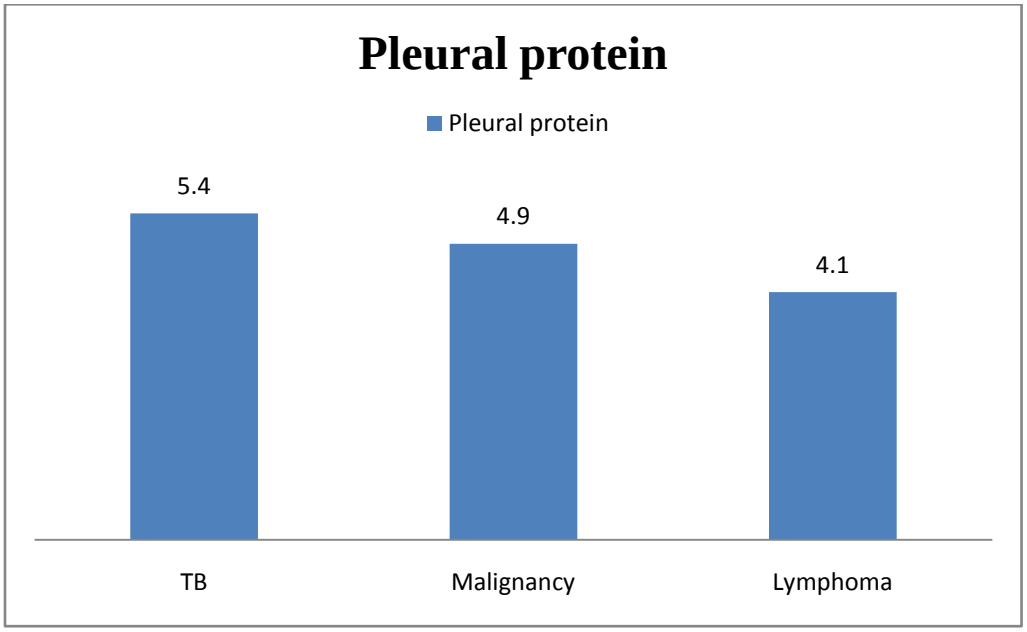

Fig 4: showing pleural fluid protein levels in TB, malignancy & lymphoma

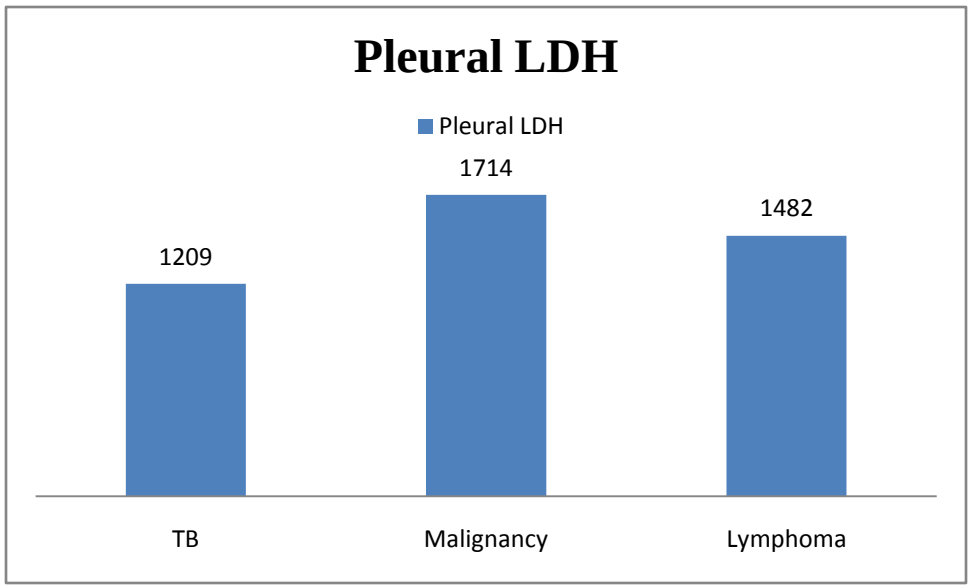

Fig 5: showing pleural fluid LDH levels in TB, malignancy & lymphoma.

Aetiology of pleural effusion (Fig 6)

Among the 154 patients who were included in the study, 68 patients had tuberculosis, followed by 31 patients with malignancy which included primary from lung malignancy as well as other sites. Seven patients had lymphoma based on pleural biopsy. Four patients had effusion secondary to mesothelial tumour which included 2 benign mesothelial proliferations and 2 mesothelioma. Two cases with eosinophilic effusion

which was secondary to parasitic infestation-H.nana and Churg Strauss syndrome. In one patient, the cause for pleural effusion was fibrous tumour. Thirty six (23%) patients had no diagnosis despite pleural fluid studies and pleural histopathology.

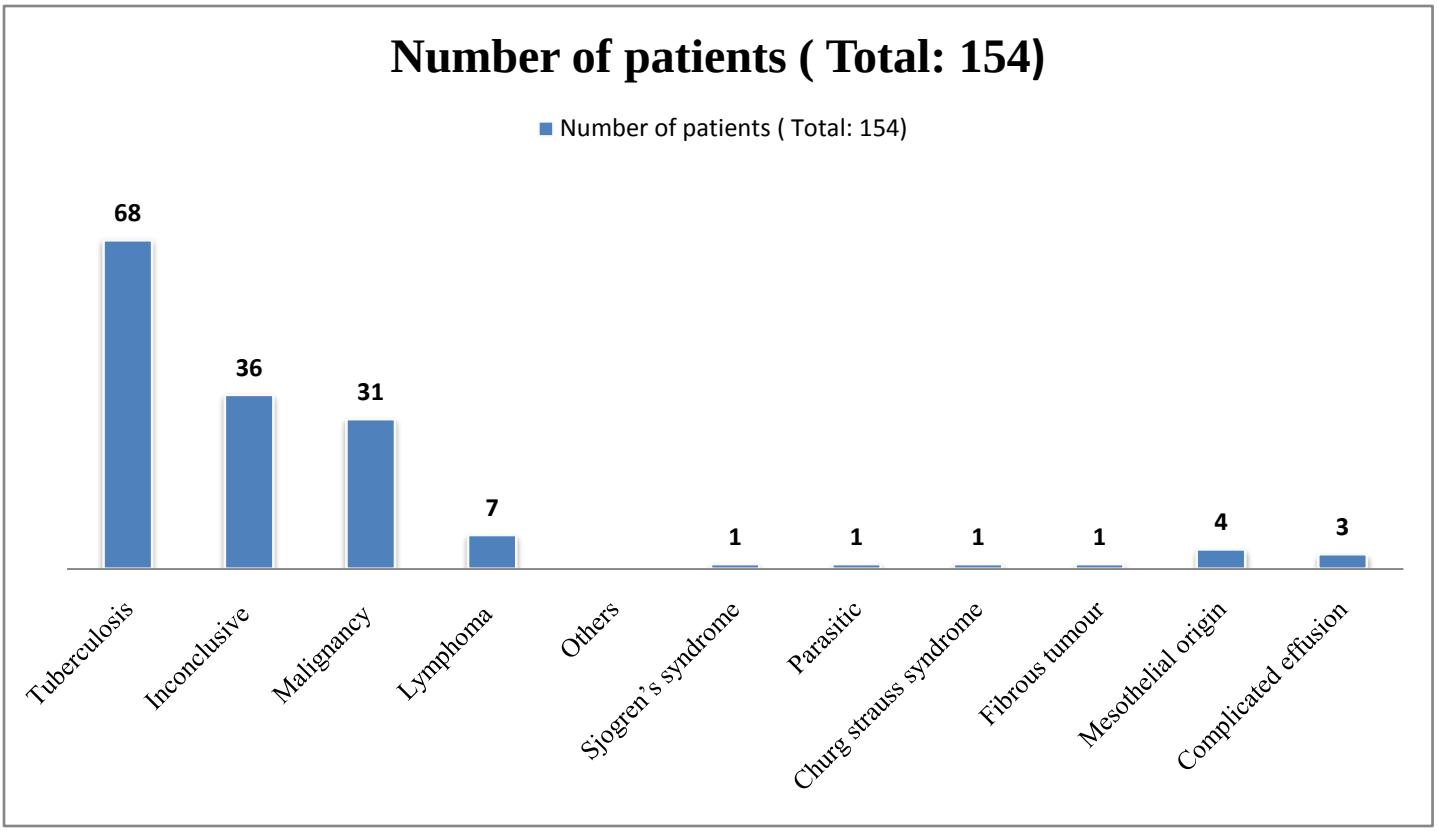

Fig6: showing the aetiology of pleural effusion

MTB yield on pleural tissue and fluid cultures: (Fig 7)

All patients who underwent pleural fluid aspiration and pleural biopsy, the pleural samples were sent for cultures. 17 patients had pleural tissue culture growth and only 4 patients had pleural fluid growth for Mycobacterium tuberculosis among the 68 patients with tuberculous pleural effusion. Yield from AFB smear was extremely rare, occurring in only few patients.

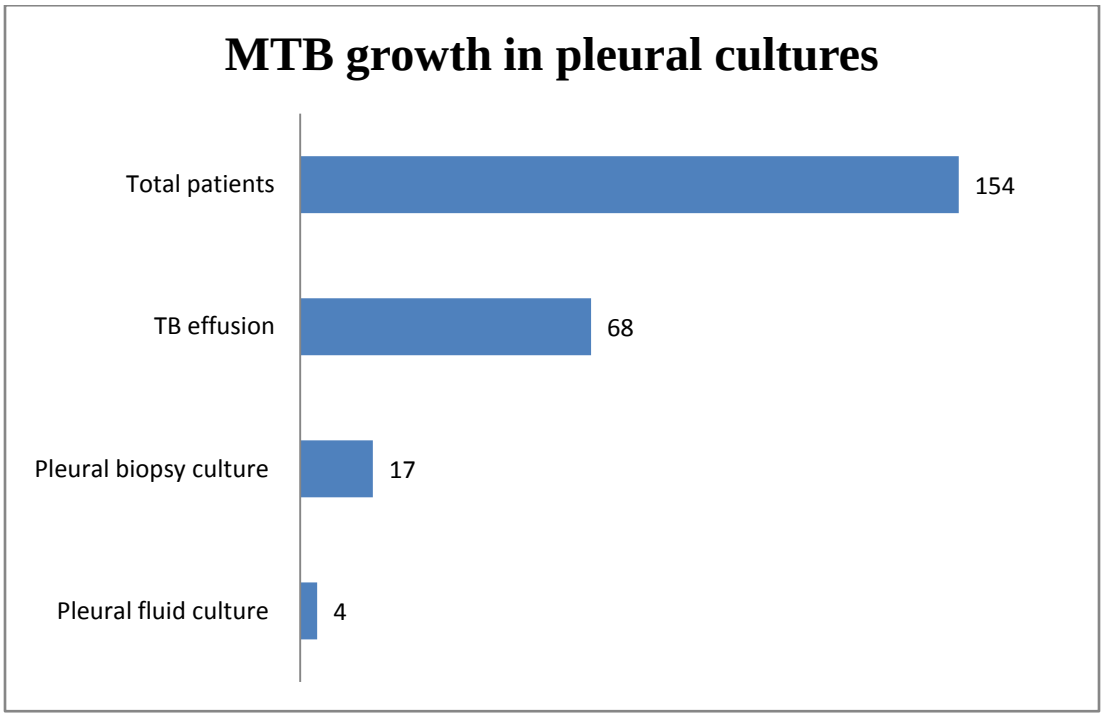

Fig 7: Showing pleural fluid and biopsy culture yield in the diagnosis of tuberculous pleural effusion.

Cytology yield in malignancy (Fig 8)

Figure 6 shows the yield of cytology in diagnosis of malignancy. 14 samples yielded the diagnosis of malignancy among the 31 subjects (45%) with malignancy.

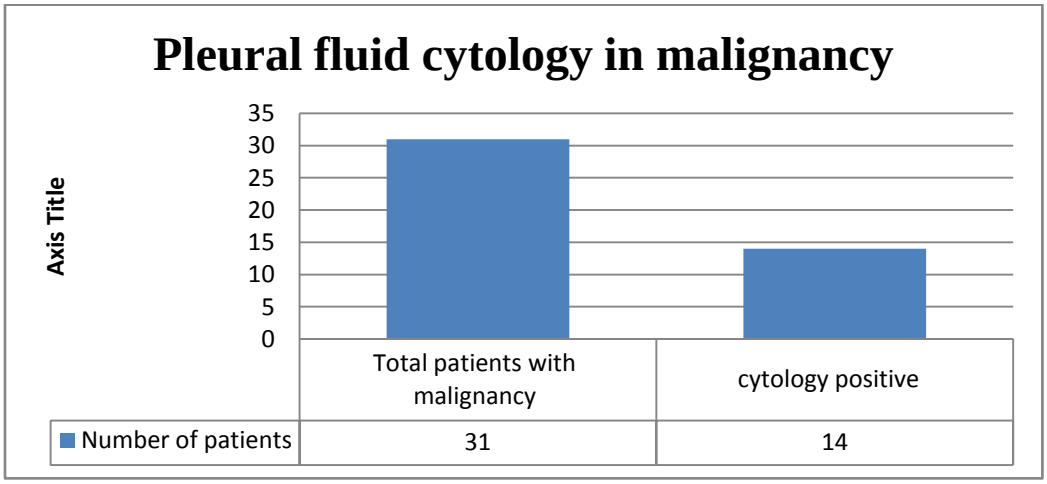

Fig 8: showing the diagnostic yield of pleural fluid cytology

Pleural fluid biomarkers in pleural tuberculosis

(Table 6, Fig 9, 10, 11)

Adenosine deaminase (ADA):

Adenosine deaminase was estimated in the pleural fluid samples. A cut off of 24U/L and above was taken as positive and a 2x2 table was used for analyzing the sensitivity and specificity. ADA had a sensitivity of 70.4% and a specificity of 71.1 %. The positive predictive value is 63.3 % and negative predictive value is 77.1%. The receiver operating curve (ROC) showed the area under the curve of 0.760

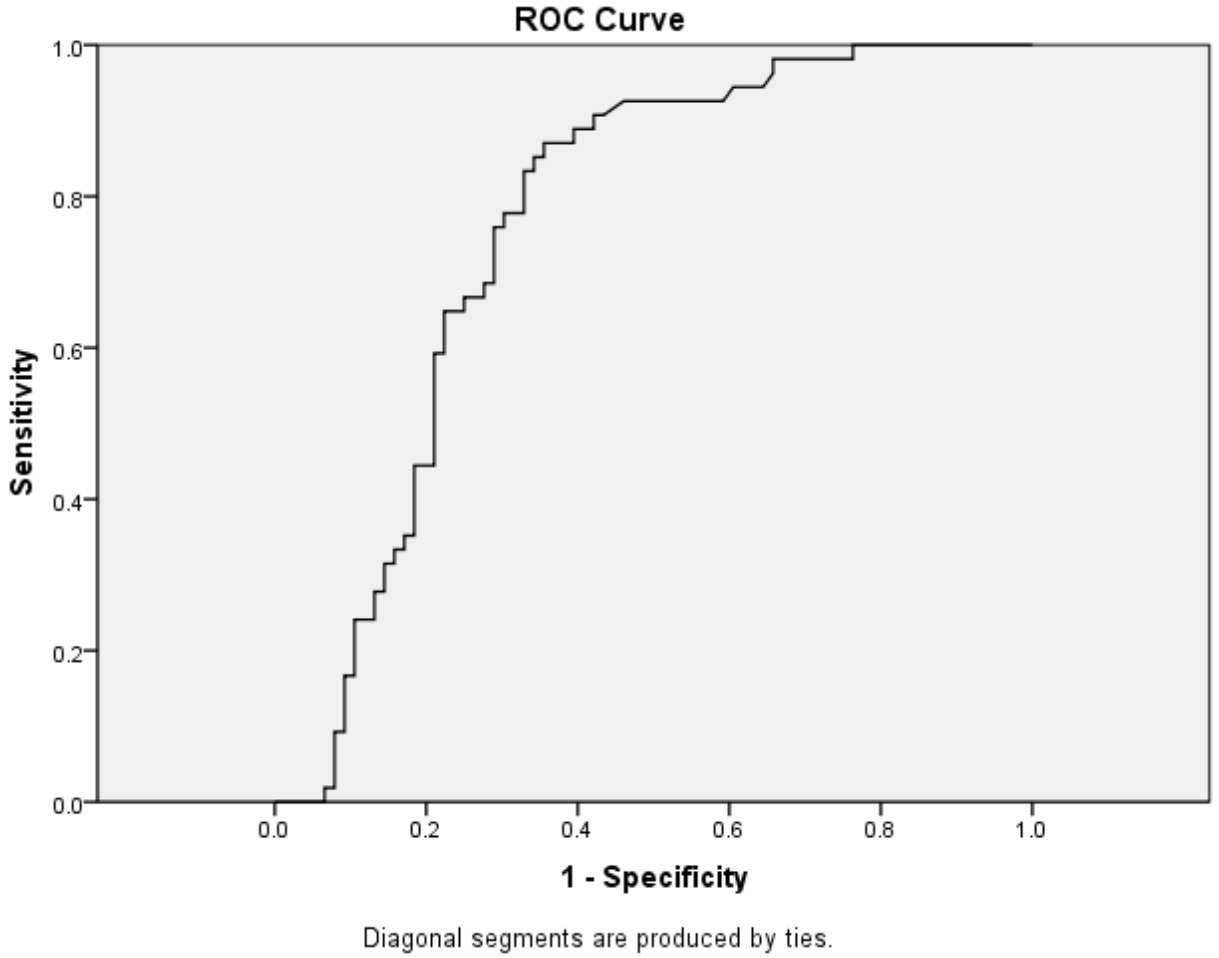

Figure 9: Showing the ROC curve for ADA

Adenosine deaminase 2(ADA2):

ADA2 is a subtype of ADA which was studied. Using a cutoff of 14U/L, ADA2 had a sensitivity of 78 % and a specificity of 62.7% for diagnosing pleural tuberculosis. The positive predictive value was 60.9% and the negative predictive value was 79.2%. The ROC for

ADA2 showed the area under the curve of 0.706. The cut off 12.6U/L is taken with a sensitivity of 80% and sensitivity of 63%.

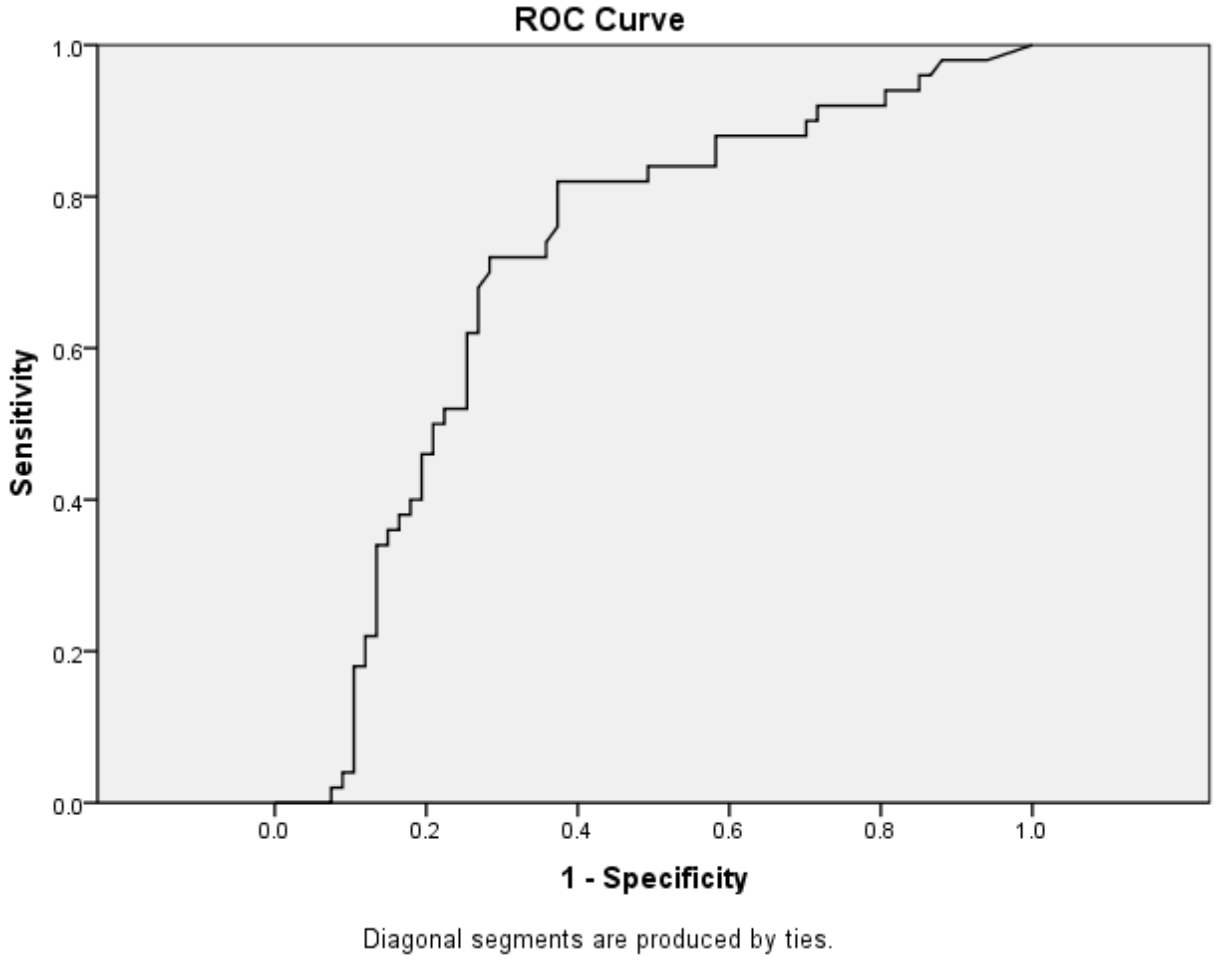

Figure 10: showing ROC for ADA2

Interferon gamma:

Using a cutoff of 2 IU/L, Interferon gamma had a sensitivity of 82.1 % with specificity of 86.2%. The positive and negative predictive values were 82.1 and 86.2 % respectively. The ROC for Interferon gamma showed the area under curve of 0.881. Using a cut-off of 3.12 IU, the sensitivity and specificity are 80% and 87% respectively.

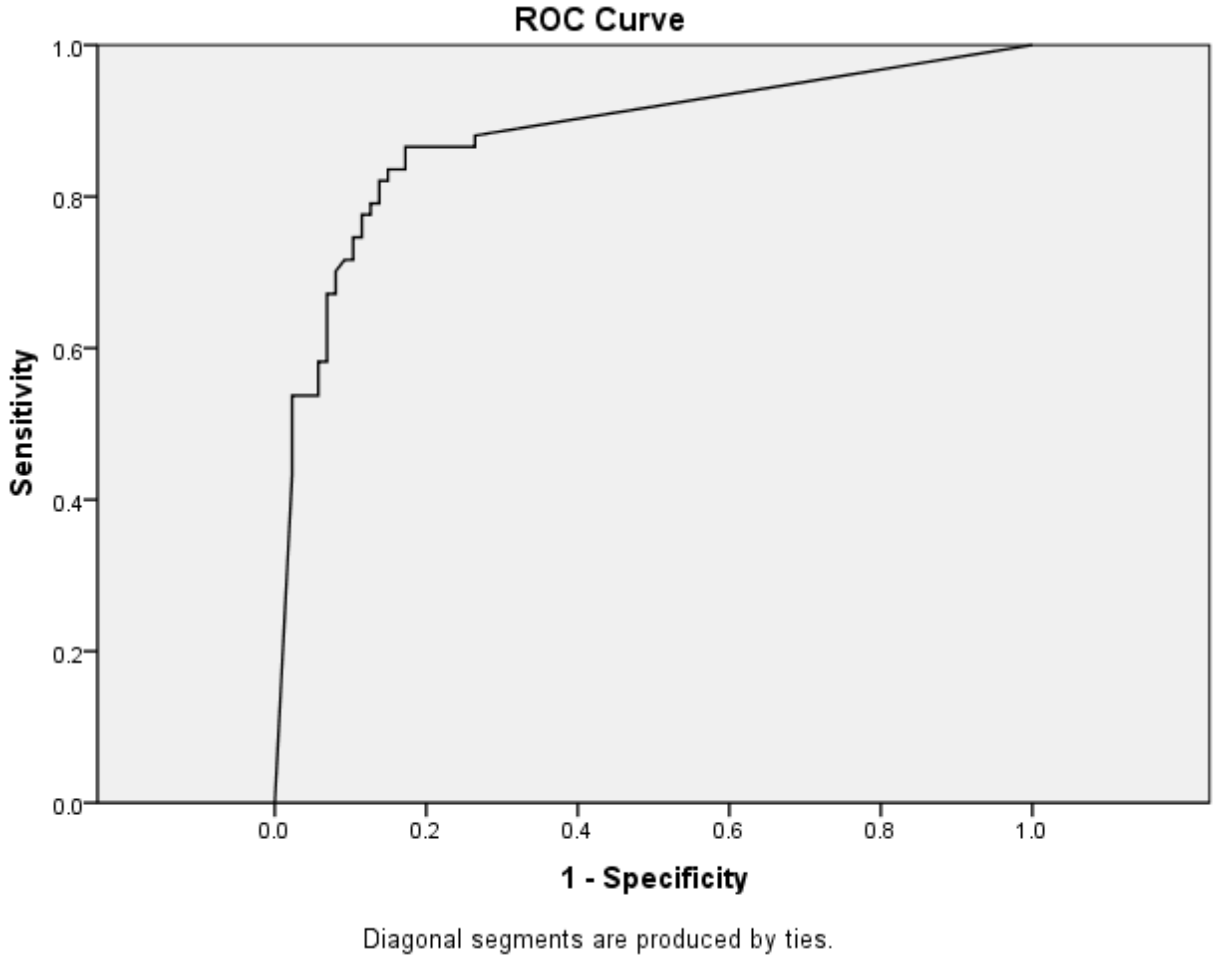

Fig 11: showing the ROC for Interferon gamma

Biomarkers	Sensitivity %	Specificity %	AUC	Cut-off U/L
ADA	70.4	71.1	0.76	24
ADA2	78	62.7	0.706	12.6
Interferon gamma	82.1	86.2	0.881	3.12

Table 6: showing the sensitivity, specificity, area under the curve (AUC) and cut-off values for biomarkers- ADA, ADA2, and Interferon gamma.

Combination of biomarkers:

The combination of biomarkers and their sensitivity and specificity were studied. When ADA was combined with ADA1/ADA ratio of ≤0.42, the sensitivity improved from 70.4% to 79.6% but the specificity declined from 71.1 % to 39.2%.

The combination of ADA, ADA1/ADA ratio with Interferon gamma improved the sensitivity from 70.4% to 93.8% but it lacks specificity 35%. A combination of ADA and ADA2 had a better sensitivity of 80% but lacks specificity 62.7%.

The lymphocyte neutrophil ratio showed sensitivity of 87.5% but lacks sensitivity, 16.5%. The combination of tests improved the sensitivity but failed to improve specificity.

Biomarkers	Sensitivity	Specificity	PPV	NPV
ADA+ ADA1/ADA	79.6	39.2	48.9	72.5
ADA+ ADA1/ADA+ Int γ	93.8	35	53.6	87.5
ADA+ ADA2	80	62.7	61.5	80.8

Table 7: showing combination of biomarkers and their sensitivity, specificity, positive and negative predictive value in the diagnosis of TB pleural effusion.

Pleural fluid biomarkers in disease conditions:

The pleural fluid biomarkers were compared in tuberculosis, malignancy and lymphoma. The mean ADA levels in tuberculosis was high, it was low in malignancy and very high in lymphoma.ADA2 levels followed a similar trend.

Interferon gamma in TB patients was high and was low in both lymphoma and malignancy.

Biomarkers	ADA U/L	ADA1 U/L	ADA2 U/L	Interferon gamma IU
Tuberculosis	47.0	15.9	34.7	18.3
Malignancy	15.4	7.6	10.6	1.47
Lymphoma	131.3	31.0	22.3	1.46

Table 8: showing pleural fluid biomarkers levels in tuberculosis, malignancy and lymphoma.

6. Discussion:

Demographic profile:

During the period between May 2012 and July 2013, a total of 154 patients were included in the study. The study population had a male predominance of 74%. The mean age of patients was 44.8 years. Among the 154 patients, 146 patients had unilateral pleural effusion. Bilateral pleural effusion was seen in eight patients. The aetiology for bilateral pleural effusion was further analyzed and three patients had tuberculosis, one patient had adenocarcinoma, one patient had Sjogren's syndrome and in three others, diagnoses were not conclusive. Tuberculous pleural effusion could also be bilateral (36). An article published in the year 1945 by E.Montushi, in his series of 601 patients, 6 percent of bilateral pleural effusion was due to tuberculosis(36).

One patient with pleural effusion had HIV infection. Among the 154 patients with pleural effusion, 37% of patients didn't have sputum examination for acid fast bacilli (AFB) and two patients

were AFB positive. Sputum examination should be performed in suspected tuberculous pleural effusion. A study by Chaudhuri et al(37) looking at sputum examination in tuberculous pleural effusion showed a pickup rate of 22.22%. Another study looked at the yield of induced sputum in diagnosing tuberculous pleural effusion showed 52% yield on sputum cultures(38)(5)

Among the 68 TB effusions, 53 were men and 15 were women. The male: female ratio was 3.5:1. A higher male: female ratio of 1.6:1(39)was also seen in a Spanish study. A scoring system framed for diagnosis of TB pleural effusion used male gender as one of the variable indicating that TB effusion is more among men(40).

Age distribution in disease condition:

The mean age of patients who had TB pleural effusion was 39.13 years. The minimum age with TB effusion was 17 years and maximum age 85 years. The mean age for malignancy and lymphoma are 53 years and 47 years respectively. The above data states that tuberculous pleural effusion is more common in the younger population and malignancy more common in the older population.

Valdes et al(39) showed that tuberculous pleural effusion is more common in younger age group (age less than 40 years), malignancy is more common among age group of more than 50 years and tuberculosis is the most common cause for pleural effusion followed by malignancy and heart failure.

Pleural fluid cellular characteristics:

The pleural fluid characteristics showed a large proportion of patients about 80% had lymphocyte predominant pleural effusion fewer patients had neutrophilic effusion and a small proportion ; eosinophilic pleural effusions. The common causes for lymphocytic pleural effusion are; tuberculosis and malignancy and the commonest cause for neutrophilic effusion is parapneumonic effusion(41).

Ten patients had eosinophilic pleural effusion. The causes of eosinophilic pleural effusion in our study were- adenocarcinoma - two patients, T-cell lymphoma, tuberculosis, Churg-Strauss syndrome, Sjogren's syndrome, H.nana infestation were the causative factors in one patient each, and in three patients the aetiology was uncertain. Krenke et al(42)in his study showed that malignancy is the commonest cause for eosinophilic effusion, followed by infections, unknown, post traumatic and miscellaneous causes.

Pleural fluid bio-chemical parameters:

The pleural fluid biochemical parameters mainly pleural fluid protein and lactate dehydrogenase (LDH) were analyzed in each disease conditions. In our study, high protein levels with a mean of 5.4mg/dL were seen in tuberculous pleural effusion followed by malignancy and lymphoma with a mean level of 4.9mg/dL and 4.1mg/dL respectively.

An Indian study revealed pleural fluid protein more than 3gm in 94.34%(23) of tuberculous pleural effusion. In another study which looked at pleural fluid analysis in diagnosing TB pleural effusion, a combination of three parameters- pleural fluid protein ≥5g/dl, lymphocytes >80% and ADA > 45U/l , had a specificity of 100% and sensitivity of 34.9%(43)

High levels of lactate dehydrogenase(LDH) levels are found in lymphoma and malignancy. Tuberculous pleural effusion had a mean LDH value 1209. A study by Ernam et al(44) showed that pleural fluid LDH levels were very high in parapneumonic

effusions followed by malignancy and then in tuberculous pleural effusion. Therefore, pleural fluid LDH can be elevated in many conditions including malignancy, lymphoma, tuberculosis and other conditions and is a non specific marker for making a specific diagnosis.

Aetiology of pleural effusion:

The aetiology of pleural effusion among the 154 patients was studied. Sixty eight patients (44%) had tuberculous pleural effusion, followed by 36 patients (23%) with pleural effusion of uncertain aetiology, followed by malignancy in 31 patients (20%). Seven patients had lymphoma; four patients had effusion secondary to mesothelial neoplasm - 2 benign mesothelial proliferations and 2 mesothelioma. A small subset of patients had effusion due to rare causes like Sjogren's syndrome, Churg Strauss syndrome, parasitic infestation and fibrous tumour. The above data suggests that the common cause for pleural effusion is tuberculosis followed by malignancy. The observation by Valdes et al(39)

showed that tuberculosis is the common cause of pleural effusion in an area with high tuberculosis burden followed by malignancy and cardiac failure.

MTB yield on pleural cultures:

Among the 68 patents with tuberculous pleural effusion, 17 patients (25%) had pleural biopsy culture growth and 4 patients (5%) had pleural fluid culture growth.

A study showed that pleural biopsy culture had a better yield than pleural fluid culture, 62% versus 12% respectively(38) The British thoracic society guidelines quotes a 10-20% smear positivity rate on pleural fluid and 25-50% for pleural fluid cultures(41)

An article by Trajman et al(26) showed that the yield of pleural fluid microscopy for AFB is <5% and pleural fluid cultures had a low sensitivity of 24-58%. Overall, pleural fluid culture is less useful and pleural biopsy culture had better yield.

Pleural fluid cytology yield in diagnosis of malignancy:

Among the 154 patients, 122 pleural fluid samples were sent for cytological examination. Fourteen samples were positive for malignant cells supporting the diagnosis of malignancy. Our study had a pleural fluid cytology yield of 45%.

A retrospective analysis of 281 patients with malignant pleural effusion was studied and pleural fluid cytology yielded the diagnosis in 57.6% (45) Another study looked at the yield of pleural fluid cytology showed a yield of 65%(46) The yield of pleural fluid cytology is variable and largely depends on skill and interest of the cytologist and the tumour type(41)

Pleural fluid biomarkers in pleural tuberculosis:

In this study, we studied the role of pleural fluid biomarkers in the diagnosis of tuberculous pleural effusion. Pleural biopsy histopathology and cultures are the currently available gold standards for the diagnosis of tuberculous pleural effusion. The limitations for closed pleural biopsy are its complications like pain,

haemothorax, pneumothorax and very rarely death due to haemorrhage(7) To overcome these complications, we study the role of biomarkers in diagnosing TB effusion. In this study, we looked at three biomarkers-ADA, ADA2 and Interferon gamma.

a. Adenosine deaminase (ADA):

ADA is a biomarker which is found elevated in TB pleural effusion. A cut off of ADA as 24 U/L was taken and the sensitivity and specificity were analyzed. The cutoff of 24 was taken as per the CMC biochemistry lab standards. The sensitivity of pleural fluid ADA in the study was 70.1% and specificity was 71.1%. Which was low compared to other studies. Kelam et al(47) showed a high sensitivity (90%) and a specificity of 50% in his study. In a meta analysis of 63 studies reported, a sensitivity of 92% and specificity of 90% with a cut off of 40u/l(21).

The drawback of ADA is it lacks specificity. ADA can be elevated in many other conditions like parapneumonic effusions, empyema, and lymphoma, infections like brucellosis and in rheumatoid

arthritis thereby lacking specificity. ADA can be used as a screening tool because of its high sensitivity in tuberculous pleural effusion.

b. Adenosine deminase 2 (ADA2):

ADA has two isoenzymes ADA1 and ADA2. ADA2 is found in macrophages and is released when stimulated by intracellular microorganisms. Our study revealed, ADA2 had a better sensitivity than ADA but it also lacks specificity. By analyzing the ROC with AUC of 0.706, the best cutoff value for ADA2 was 12.6U/L. Using a cutoff of 12.6U/L, the sensitivity of ADA2 was 80% and specificity was 63%. Valdes et al(48) studied ADA isoenzymes and reported a sensitivity of 100% and specificity of 96% in TB pleural effusion.

c. Interferon gamma:

Interferon gamma is released by activated CD4 +T lymphocytes. The cytokine is released when there is increased mycobactericidal activity by the macrophages. In our data, Interferon gamma had a sensitivity of 82.1% and a specificity of 86.2%. Interferon gamma had a better sensitivity and specificity compared to ADA and ADA2. By analyzing the ROC, the best cut off for Inteferon gamma was 3.12IU. Using a cutoff 3.12 IU, the sensitivity was 80% and specificity was 87%.

A meta analysis of 22 studies on Interferon gamma showed a mean sensitivity of 89% and a mean specificity of 97%(21). A study looked at ADA and Interferon gamma levels in the diagnosis of TB pleural effusion. The sensitivity and specificity of ADA was 86% and 74% respectively whereas 100% sensitivity and 100% specificity for Interferon gamma(49).

In our study, Interferon gamma had a better sensitivity and specificity compared to ADA and ADA2.

d.Combination of biomarkers:

A combination of biomarkers and its role in diagnosing TB pleural effusion was studied. When combining ADA and ADA1/ADA ratio, the sensitivity was 79.6% and specificity was 39.2%. When ADA with ADA1/ADA and interferon gamma was combined, the sensitivity improved to 93.8% but the specificity was only 35%.

When ADA and ADA2 was combined, the sensitivity was 80% and specificity was 62.7%. In our observation, combination of tests improved the sensitivity but failed to improve specificity.

Pleural fluid lymphocytosis is one of the feature of tuberculous pleural effusion(41). Pleural fluid lymphocyte neutrophil ratio was calculated; the ratio had a sensitivity of 87.5% and a specificity of 16.5%.

Valdes et at(48) studied the combination of ADA >47U/L , ADA2 >40U/L and ADA1/ADA ratio <0.49 with a sensitivity of 100% and specificity of 97%. They concluded that ADA2 was a better marker than ADA. In effusions with high ADA levels; the ratio of

ADA1/ADA helps to differentiate tuberculous effusion from para-pneumonic effusion, but fails to discriminate malignant effusions.

A study by Keng et al(50) looked at combination of ADA ≥40 IU/L and Interferon gamma ≥ 75pg/mL had 100% specificity.

Burgess et al(51) studied the combination of ADA with lymphocyte neutrophil ratio and found a sensitivity of 88% and specificity of 95%.

Pleural fluid biomarkers in disease conditions:

The pleural fluid biomarkers-ADA, ADA1, ADA2 and interferon gamma are analyzed with diseases like tuberculosis, malignancy and lymphoma. The ADA values are found high >100U/L in lymphoma and low in malignancy. In tuberculosis, the mean ADA level was 47U/L. A Meta analysis of 63 studies on ADA showed a sensitivity and specificity of 92% and 90% with cut off value 40U/L(21).

ADA1 and ADA2 levels were elevated in lymphoma and tuberculosis and lower levels are seen in malignancy. Interferon gamma levels are elevated in tuberculosis.

A combination of ADA >40U/L with Interferon gamma >3.12IU can improve the diagnostic yield in tuberculous pleural effusion.

7. Limitations of the study:

One of the limitations of this study is the small sample size. We recruited 154 patients and among them 68 patients had tuberculosis. Studies with large sample size are required to further analyze the role of biomarkers in tuberculous pleural effusion. Thirty six patients had inconclusive biopsy results and were labeled inconclusive. Further evaluation is required to find the cause for effusion and make correlation with the biomarkers.

8. Clinical implications:

The findings of the study have potential clinical implications:

1. Among the three pleural fluid biomarkers, Interferon gamma has a good sensitivity and specificity which can be clinically utilized in diagnosing TB pleural effusion.

2. A combination of ADA >40IU/L with interferon gamma >3.12IU can improve the diagnostic yield in tuberculous pleural effusion and these markers can be utilized in centers where there are limitation of resources for pleural biopsy and culture methods.

9. Conclusion:

Tuberculous pleural effusion is one of the common causes for pleural effusion with a diagnostic challenge. The pleural fluid biomarkers, combination of tests and scoring system have an important role in diagnosing TB effusion but are not sufficient to replace the conventional gold standard methods for diagnosis.

10. Areas of future work:

a. A further study with large sample size is required to validate on pleural fluid biomarkers.
b. A scoring system by combining biomarkers with clinical parameters is required to improve the specificity.
c. Role of molecular diagnostic methods like Gene Xpert in TB pleural effusion.

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Annexure:

I. Patient information sheet

II. Consent form

III. Data sheet

IV. IRB clearance

I. PATIENT INFORMATION SHEET:

Contact Information:

Dr. A. Ashwin Oliver

Department of Pulmonary medicine

Christian medical college, Vellore- 632004

Purpose of Subject information & Consent form:

The purpose of this form is to inform you about this research study. If you sign this form, it means that you have agreed to take part in this study. The form describes the purpose, procedures, benefits, risks and side effects of the research study. It may contain words that you do not understand. Please ask the study doctor or personnel to explain any words or procedures that you do not clearly understand. You may also want to discuss this with your family and/or friends. You may refuse to take part or withdraw from this study at any time. This will not affect your medical care at the Christian Medical College (CMC) hospital.

Introduction:

You are being asked to participate in this study because your doctor thinks you may have signs and symptoms of pleural tuberculosis (TB). This is a research project conducted by the Department of pulmonary medicine, Christian Medical College, Vellore, India. The research study evaluates the performance of a new simple, rapid test to detect TB in the pleura (lining of the lungs).

As you may be aware, tuberculosis is an infection that mostly affects the lungs. However, TB can also affect other organs, including the lung lining, brain, abdomen, bone and joints, and lymph nodes and sometimes also several organs at the same time. TB is very common in India. It is a major cause of death and disability. Consequently, there is a need to develop and evaluate new or improved tests.

Purpose of the study:

The purpose of the study is to compare the results of a new simple, rapid test for pleural TB against the best tests that are currently in use. We plan to perform the new as well as the currently available diagnostic tests in persons with suspected TB disease in their lung lining at the Christian Medical College Hospital, Vellore.

Procedures:

You will be asked to undergo the following tests, if your doctor suspects TB of the lung lining in you. All of these tests are done routinely outside of the research study as well. These tests include:

Pleural tap and collection of a pleural tissue specimen:

A trained, specialized physician (a chest doctor) will do the pleural tap and biopsy. A needle will be inserted in between your ribs into the area between your chest wall and your lung (the pleural space). The doctor will draw 10 cc of fluid (2 teaspoons) from your chest and will take a small tissue sample from the inner lining of your

chest wall. Again this is a routine test if TB of the lung lining is suspected.

The following tests will be done on the pleural fluid/biopsy sample:

a. Smears to be examined under a microscope

b. Culture to grow TB bacteria

c. Pleural tissue sent for histo-pathological examination

d. Left-over pleural fluid will be stored in a freezer for possible further testing

Your pleural fluid specimen will be used to perform this new test, which is the only test that is not usually performed.

Potential Risks & Discomforts:

This study does not involve any new drugs or treatment. It involves adding additional tests for the diagnosis of TB to the tests currently used. The risks are mainly related to testing procedures (pleural tap

and biopsy) that are done routinely for the diagnosis of your condition outside of a trial as well. The most common problems from a pleural tap include temporary soreness, bruising, and discoloration of the chest area where the needle is inserted. Some individuals may feel dizzy or faint during the pleural tap procedure. In rare cases there may be injury to the nerves, resulting in shoulder or neck pain. In very rare cases there may also be damage to the lung or abdominal organs resulting in bleeding or in lung collapse, which might necessitate surgery or placement of a chest tube.

Potential benefits:

Your participation may provide the medical community with information about whether a new, simple rapid test for pleural TB will work or not. You are assured the standard of care in terms of TB diagnosis whether you participate in this study or not. If you are found to have TB, you also will receive the standard of care. You will be referred to your usual health care provider at the CMC

hospital, where free treatment will be provided. A highly effective treatment for TB exists, and your health care provider will discuss this treatment with you, and initiate therapy following the guidelines of the Indian National TB Control Programme. If you are infected with HIV, you will also be referred for treatment. If other illnesses are identified, you will be referred for further evaluation and care.

Potential Future Uses and Storage of the Samples:

We are asking your permission to store a sample of your pleural fluid. Some of the fluid that is taken from your chest may be frozen and stored (banked) and used for future research related purposes for TB. The samples will not be sold, or used for any commercial purpose. They can be stored up to 2 years, after which the samples will be destroyed. It remains your right to ask for the destruction of your samples at any time. If you want your samples to be removed from the bank, you should contact Dr. A. Ashwin Oliver.

Confidentiality:

All of the information that we obtain during this research will be kept confidential. No one other than the study investigators will know your identity from our files. The results of this research study may be presented at meetings or in scientific publications; however, your identity will not be disclosed in any of these presentations. As part of this study, we will request your authorization to access information from your medical files from the CMC hospital for your TB related evaluation, including details of other TB investigations (if any) and TB related outcomes. The ethics committee of CMC Vellore may also access your medical files. The samples that will be banked for future research purposes will not contain your name on them. They will simply have a code number and they will be stored in a freezer for 2 years. Only a few staff members of the research team will have access to the sample.

Voluntary participation & withdrawal:

It is entirely your choice whether or not you participate in this research study. If you decide not to participate, your current and future medical care at the CMC will not be affected by this choice. You may decide to withdraw from this study at any time. Your participation in this study may be terminated by you, or your study investigators.

Legal Rights:

You are not waiving any of your legal rights by participating in this study or by signing this consent form. This includes, for example, the right to seek damages under law for any research related injury.

II. SUBJECT INFORMED CONSENT:

1. I understand that this is a research study.

2. I have read all the pages of the consent form. The research personnel have explained the information and procedures involved in the study. I have had the opportunity to ask questions and my questions have been answered satisfactorily. I have been given time to consider the information carefully and to decide whether or not to participate in this study.

3. I have been informed that my participation in this study is entirely voluntary and that I may refuse to participate, or withdraw at any time, without any consequences to my ongoing and future medical care at this institution.

4. I authorize the release of my medical records to the study investigators as well as the ethics committee of CMC Vellore for purposes of this study only. This authorization will be valid for a period of 5 years.

5. I understand that I will be given a copy of this informed consent form to keep for my own information, once it is signed. I have been informed that a copy of this consent form will be placed in my medical chart so that health care providers at this institution will know that I am participating in a study and what is involved.

6. I understand that I do not give up any of my legal rights by signing this form nor am I freeing the investigators, sponsors, or the health establishment where the study takes place from their civil and professional responsibilities.

7. I understand that the samples I provide will be used for the purpose of this research and other related research.

1. My signature below indicates that I voluntarily agree to take part in this study.

_____ _____

_____

Subject's signature Name (in block letters)

Date

_____ _____

_____

Signature of Person

Date

Administering Informed Consent Name (in block letters)

I confirm having met with the subject at the time of enrolment and have I have answered his/her questions about this study.

Investigator or Project coordinator's signature:

Name (in block letters) Date

Office of the Addl. Vice Principal (Research)

Christian Medical College,
Vellore 632 002

Ref: Res/01/2012

May 7, 2012

Dr. Ashwin Oliver
Department of Pulmonary Medicine
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
To study the diagnostic accuracy of pleural fluid biomarkers – ADA, ADA2 & interferon gamma Vs conventional methods – pleural biopsy and cultures in the diagnosis of pleural tuberculosis.
Dr. Ashwin Oliver, Pulmonary Medicine, Dr. DJ Christopher, Dr. Richa Gupta, Pulmonary
Medicine, Dr. Victoria Job, Clinical Biochemistry.

Ref: IRB Min. No. 7733 dated 04.01.2012

Dear Dr. Oliver,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "To study the diagnostic accuracy of pleural fluid biomarkers – ADA, ADA2 & interferon gamma Vs conventional methods – pleural biopsy and cultures in the diagnosis of pleural tuberculosis " on January 4, 2012.

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released?

With best wishes,

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. B.J.Prashantham	MA (Counseling), MA (Theology), Dr Min(Clinical)	Chairperson(IRB)& Director, Christian Counselling Centre	Non-CMC
Mr. Harikrishnan	BL	Lawyer	Non-CMC
Mrs. S. Pattabiraman	BSc, DSSA	Social Worker, Vellore	Non-CMC
Dr. Jayaprakash Muliyil	BSC, MBBS, MD, MPH, DrPH(I DMHC	Academic Officer, CMC	
Mrs. Ellen Ebenezer Benjamin	M.Sc. (Nursing).	Deputy Nursing Superintendent, CMC.	
Dr. Vathsala Sadan	M.Sc. (Nursing), PhD	Addl. Deputy Dean, College Nursing, CMC.	
Dr. Gagandeep Kang	MD, PhD, FRCPath.	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Microbiology & Addl. Vice Principal (Research), CMC.	

We approve the project to be conducted as presented.

The Institutional Review Board expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent and requires a copy of the final report.

A sum of Rs 60,000/- (Rupees Sixty thousand only) is sanctioned for 18 months.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

May 7, 2012

Dr. Ashwin Oliver
Department of Pulmonary Medicine
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
To study the diagnostic accuracy of pleural fluid biomarkers – ADA, ADA2 & interferon gamma Vs conventional methods – pleural biopsy and cultures in the diagnosis of pleural tuberculosis.
Dr. Ashwin Oliver, Pulmonary Medicine, Dr. DJ Christopher, Dr. Richa Gupta, Pulmonary Medicine, Dr. Victoria Job, Clinical Biochemistry.

Ref: IRB Min. No. 7733 dated 04.01.2012

Dear Dr. Oliver,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "To study the diagnostic accuracy of pleural fluid biomarkers – ADA, ADA2 & interferon gamma Vs conventional methods – pleural biopsy and cultures in the diagnosis of pleural tuberculosis " on January 4, 2012.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Cvs of Drs. Ashwin Oliver, DJ Christopher, Richa Gupta, Victoria Job
3. A CD containing documents 1 – 2

The following Institutional Review Board (Ethics Committee) members were present at the meeting held on January 4, 2012 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore- 632002.

age	sex	hno	sput	hiv	sideffu	serprot	serldh	pltc	plpoly	pllymph	pleosin	plprot	plldh
72	1	293690F	3	2	1	4.9	243	4320	2	98	0	3.9	306
45	1	292928F	2	2	2	6.8	1134	1600	17	36	47	5.4	3716
59	1	286652F	2	2	1	7.3	629	220	6	94	0	1	1093
30	1	285212F	3	2	2			290	4	96	0	5	1108
55	1	287404F	2	2	1	7.1		1280	93	7	0	6.2	2312
53	1	289691F	3	2	1	9.2							
35	1	283057F	2	2	1	7.6		1900	24	57	19	4.9	485
28	1	287346F	2	2	1	7.4	839	4000	3	97	0	5	519
68	1	285608F	2	2	1			850	5	95	0	5.5	458
31	2	290883F	2	2	1	6.7	918	1100	12	88	0	5.4	5012
28	2	695079D	2	2	2	7.3		1560	18	82	0	4.8	369
31	2	300468F	2	2	2	5.7		50	26	74	0	1.3	124
57	1	293743F	2	2	1			2100	6	94	0	5.1	3393
31	1	178072F	2	2	3	8.1		35	76	24	0	5.8	1183
48	1	299377F	3	2	2	6.8	528	1500	19	71	10	6.1	4734
59	1	684173D	2	2	1							4.8	485
54	1	704400A	3	2	1	7.8	348	3950	4	96	0	5.9	545
24	2	331382F	2	2	2	8.5	407	960	3	97	0	6	998
32	1	332849F	2	2	2	5.5	2319	4800	2	98	0	3.6	5200
64	1	318381F	2	2	1	5.1		1800	53	47	0	0.7	96
41	1	329704F	2	2	1	7.6		3300	3	97	0	5.7	523
49	1	263095F	3	2	1	7.6	353	1300	2	98	0	5.6	530
53	1	337272F	3	2	1			320	12	87	1	5.4	1071
41	1	303063F	2	2	1	7.8		3200	3	97	0	4.7	245
17	1	319358F	2	2	2	8.1		480	25	75	0	5.6	1592
52	1	340889F	3	2	2			4800	2	98	0	6	517
65	1	138476F	2	2	1	9.1		3600	2	98	0	6.5	344
29	1	337053F	3	2	2	9.7	544	6500	5	95	0	6.4	862
52	2	337289F	2	2	1	7.9		6400	2	98	0	5.4	194
55	1	340493F	2	2	2	7.5	1668	3350	82	18	0	5.5	1400
23	1	323129F	3	2	1			800	52	48	0	6.1	881

61	1 825833D	2	2	2	8.3		130	15	85	0	2	184
57	1 352889F	2	2	1	7.4	452	1400	7	92	1	5.2	706
44	1 007118F	1	2	1	6	555	1100	20	80	0	4.3	554
22	2 317887F	2	2	2	7.8		17947	92	8	0	5.6	6850
85	1 333461F	2	2	2	6.5	574	960	2	98	0	4.3	816
74	1 232062B	3	2	2	7.5	394	640	66	28	6	1.7	7640
55	1 347626F	2	2	2	7.1	364	280	80	18	2	5.4	3889
66	1 310685F	3	2	1			960	9	91	0	4.8	1933
17	2 355737F	3	2	1	7.1	378	2500	2	98	0	6.4	284
74	1 518121C	3	2	1	7.3	816	15200	81	19	0	4.9	1015
38	1 398575F	3	2	1			1600	4	95	1	3.2	303
30	1 388888F	2	2	1	7.5		1700	1	99	0	5.9	545
42	2 391131F	3	2	2			350	19	80	1	5.2	416
37	1 389668F	3	2	2	7.9		2900	3	96	1	5.3	497
51	2 061675F	3	2	1	5.8		2200	94	6	0	3.2	713
44	1 377109F	3	2	2			8500	2	98	0	5.8	472
49	2 441218F	2	2	1	7.7	457	730	2	97	1	4.6	468
40	1 446006F	2	2	1	9	605	1800	92	7	1	6.6	10600
58	1 824551D	2	2	1	6.8		640	4	96	0	3.8	373
35	2 369998F	2	2	1		486	1800	3	97	0	6	523
66	1 041887F	3	2	1	5.2	322	13500	2	98	0	3.6	295
23	1 440952F	2	2	3	8.5	502	210	8	92	0	6.1	1412
46	2 032309F	2	2	1	8.1		1920	1	99	0	3.9	125
33	1 444586F	2	2	1	8.1	457	2850	5	47	48	5.4	1189
53	1 348955D	2	2	1	6.1		23100	87	10	3	5.7	1497
22	2 506081F	2	2	2	8.5		1280	6	94	0	6.3	1020
56	2 394500F	2	2	1	7.4	1325	700	10	88	2	5.4	709
20	1 403472F	2	2	2	7		1510	80	20	0	4.5	2447
18	2 429125F	3	2	2	7.4	451	5201	12	88	0	4.9	886
61	1 417737F	2	2	1			2200	5	95	0	5.5	438
23	1 440952F	2	2	3	8.5	502	3800	2	98	0	5.1	2528
32	1 388375F	2	2	2	7.9	566	2100	14	14	72	7	383

57	1 491706	2	2	2	6.1		620	16	84	0	4.2	406
30	2 341710C	3	2	2	8	335	1600	10	83	7	5.7	341
60	1 122821C	2	2	1	7.8	536	200	16	83	1	3.3	315
35	1 377659F	3	2	2			1500	2	98	0	6	487
58	1 381352F	2	2	2	7.1		1700	32	58	0	3.2	443
60	1 414379F	3	2	1			3000	22	78	0	4.8	1006
64	1 405121F	2	2	1	7.9	447	620	30	70	0	5.2	1565
29	1 337053F	3	2	2	9.7	544	185	5	95	0	6.4	1597
67	2 417916F	2	2	1			2550	4	96	0	3.8	222
28	1 445582F	2	2	2	9.1		8000	2	98	0	6.3	450
39	1 442332F	2	2	1	7.5		2915	8	92	0	5.3	638
29	1 449900F	2	2	1	7.6		12800	2	93	5	5.8	902
29	1 388713F	2	2	1	8.6		500	93	7	0	5.1	4025
50	1 449906F	3	2	1	7.3	338	260	2	98	0	4.7	437
45	2 425278F	2	2	1	8.4		110	20	80	0	5.1	1501
76	1 417269F	2	2	1	6.8	726	1050	18	82	0	4.1	1689
38	2 553883C	2	2	2	7	362	2200	2	98	0	5.2	1034
55	2 372239F	3	2	2	7.4		1100	18	81	1	6.7	9070
24	1 380645F	2	2	2	7.5						5.5	411
28	1 369267F	3	2	1	8.9		6400	2	98	0	6.7	718
73	1 380898F	3	2	1	6.2		3000	7	92	1	10.8	2037
59	1 457372F	2	2	1			140	14	84	2	5.1	537
48	1 537072D	3	2	1	10.4	403	2500	2	98	0	4	270
28	1 464552F	2	2	2	8.7		7550	1	99	0	7.3	728
30	1 469267F	3	2	2			4300	1	99	0	5.9	744
50	1 459399F	2	2	1			4300	2	90	8	6	525
39	1 459744F	2	2	1	8.2	388	1800	85	15	0	5.3	2910
46	1 850799C	3	2	1	7.5	515	2700	3	97	0	4.8	265
25	2 455531F	3	2	1	5.5	821	400	28	70	2	1.4	425
62	1 254989F	3	2	2	6.9	388	1600	18	82	0	4.4	2164
42	1 973540C	2	2	2	8.4		5100	2	98	0	5.5	361
58	1 253266F	2	2	1	6.8	525	650	16	84	0	3.6	184

50	1 227500D	2	2	3	7	453	550	15	85	0	3.8	942
65	1 172773F	3	3	2	5.6	505	1725	3	97	0	5	730
31	2 253391F	3	2	2	7.2	290	304	60	40	0	4.3	223
63	2 257398F	2	2	2	7.1		3830	6	94	0	5.7	437
58	2 249748F	3	2	2	8	417	150	3	97	0	5.1	245
25	2 261745F	2	2	1	8.5	516					6.5	959
59	1 263695F	3	2	1			3000	1	99	0	4.9	396
48	2 262051F	3	2	2	7.1		394	5	95	0	4.8	1021
57	2 269566F	2	2	1	7.5	1470	250	2	98	0	4.1	1069
70	2 337930D	3	2	1			420	15	85	0	6.7	840
61	1 257798F	2	2	1			2010	1	99	0	5.6	392
48	1 067835D	2	2	2	7.5	353	2600	2	30	68	6.1	390
34	1 284594F	3	2	1	7.4		2250	47	53	0	5.6	293
62	1 274325F	3	2	2	5.9	728	4000	1	99	0	3	355
39	1 455689F	2	1	2	8.7		600	2	98	0	6	610
61	2 464537C	2	2	1	7.7		3400	1	99	0	6.1	240
46	1 887906C	3	2	3	5.8	407	780	2	98	0	2.9	35
55	2 314312D	3	2	2	7.4	360	480	10	90	0	5.5	244
28	1 409057F	2	2	2	8.7		215	5	95	0	5.8	590
55	1 479180F	2	2	1	7.2						4.5	748
34	1 292163F	1	2	1	7.7		15910	98	2	0	0.9	19180
57	1 486392F	2	2	2			320	4	96	0	9.1	365
63	1 610765F	2	2	1	7.9		4850	4	96	0	5.5	443
27	2 615467F	3	2	1			630	5	95	0	5.3	578
70	2 605878F	2	2	3	8.1		150	17	83	0	5.6	205
46	1 612692F	2	2	2	7.3		390	95	5	0	5.4	4090
74	1 609499F	2	2	2	7.1		410	60	40	0	4.5	1905
34	1 609189F	2	2	1			2220	3	97	0	5	677
35	1 491469F	3	2	1	7.8	698	1500	1	99	0	4.4	873
71	1 602627F	2	2	2			3700	6	15	79	6.4	957
34	2 186669F	3	3	1	8.2	602	2100	1	99	0	6.2	714
19	1 184193F	3	2	1	8.7	502	650	4	96	0	4.1	553

24	1	194133F	2	2	2	8.6	684	1920	2	98	0	5.9	359
67	1	196045F	3	2	1			5200	5	95	0	5.8	474
38	2	197355F	2	2	1	8.3		4200	2	98	0	5.3	205
75	2	000048F	3	2	1	7.4	665	480	8	92	0	5.1	695
20	1	200765F	2	2	1	8		1920	4	96	0	4.8	360
57	1	199061F	3	2	2	7.8		1120	4	96	0	5.2	441
31	1	613792C	2	2	2	7.6		3000	1	99	0	4.8	647
20	2	209291F	3	2	1	8.5	441	1280	91	9	0	6.5	4495
47	1	468772D	2	2	1	7.2	240	2880	3	97	0	6.3	250
18	1	133159F	2	2	1	8.4	475	1600	2	98	0	5.3	2258
28	2	703830A	3	2	3	11.9	270	1050	4	36	60	9.9	275
51	1	219598F	2	2	1	8.2	348	2600	95	5	0	6.2	11260
29	1	218950F	2	2	2	7.2	554	6100	98	2	0	4.7	2999
21	1	841594D	2	2	2	5.3	439	1400	1	99	0	2.9	170
16	1	221380F	3	2	1	6.8	396	4850	96	4	0	4.5	13700
42	1	220865F	2	2	1	8.3	566	2600	2	70	28	5.3	336
61	1	144552F	2	2	1	6.9	520	1100	6	30	64	5.4	432
48	1	118710F	3	2	2	8.2	602	950	4	96	0	4.4	321
41	1	961357D	2	2	1	6.6	303	6400	2	98	0	5.4	535
51	1	228328F	2	2	2	6.8	463	2700	18	77	5	4.7	2359
20	1	223229F	3	2	1	6.6	271	7200	1	99	0	5.2	671
42	2	233797F	2	2	2	8.1	547	4860	2	98	0	5.3	683
35	1	234685F	3	2	1	6.2	677	1650	30	70	0	3.6	958
43	2	224605F	2	2	1	7.2		180	16	84	0	4.4	782
50	1	219604F	2	2	1	7.4	309	1210	1	99	0	5.8	289
59	1	250607F	3	2	3	6	368	25	17	78	5	3.9	265
22	1	234313f	2	2	1	7.4	357	16000	75	25	0	6.1	824

ada	ada1	ada2	int	plbiop	plflcul	biopcul	cyto	diag	diagspe
44	11.9	32.1	15.4	1	2	2	2	1	Tuberculosis
347.5	73.1	274.4	1.86	2	2	2	2	3	T cell lymphocytic lymphoma
12	3.4	8.6	1	2	2	2	1	2	Adenocarcinoma
80.7	19.2	61.5	30	1	2	2	3	1	Tuberculosis
12.4	8.4	4	1	3	2	2	2	4	Inconclusive
	60.5		4.3	1	2	2	3	1	Tuberculosis
10	5.7	4.3	1	2	2	2	1	2	Adenocarcinoma
	13		1	2	2	2	2	3	T cell lymphoma
	9		30	3	2	2	2	4	Inconclusive
	4.8		1	2	2	2	1	2	Adenocarcinoma
	10		6.8	3	2	2	2	4	Inconclusive
	0.9		1	3	2	2	2	4	Inconclusive
	14.8		1	2	2	2	1	2	Adenocarcinoma
86.9	17.6	69.3	9.3	1	2	2	2	1	Tuberculosis
19.1			8.4	2	2	2	2	2	Adenocarcinoma
7.1	2.7	4.4	1	2	2	2	1	2	Adenocarcinoma
44.4	16	28.4	6.8		2	2	2	1	Tuberculosis
71.1	36.9	34.2	30	1	2	2	2	1	Tuberculosis
331	134	197	1	2	2	2	2	3	T-cell lymphoma
10.9	4.7	6.2	1.54	3	2	2	2	4	Inconclusive
37.6	17	20.6	30	1	2	1	2	1	Tuberculosis
40.2	12.9	27.3	30	1	2	2	2	1	Tuberculosis
12.4	4.1	8.3	1	3	2	2	2	4	Inconclusive
			1.99	3	2	2	2	4	Inconclusive
42.9	0.3	42.6	30	1	2	1	3	1	Tuberculosis
	14.1		23.5	1	2	2	3	1	Tuberculosis
23.4	10.9	12.5	1	3	2	2	2	4	Atypical mesothelial proliferation
	18.9		30	1	2	1	2	1	Tuberculosis
	20		1.58	3	2	2	3	4	Inconclusive
28.7	8.9	19.8	1.28	2	2	2	2	2	Small cell carcinoma
29	14.2	43.2	9.8	1	2	2	2	1	Tuberculosis

2	1.4	0.6	1	3	2	2	2	4	Inconclusive
14.1	11.7	2.4	1	3	2	1	2	1	Tuberculosis
25.8	1.5	24.3	1		2	2	3	1	Tuberculosis
103.6	51.4	52.2	30	3	1	2	3	1	Tuberculosis
19.7	11.7	8	12.6	1	2	1	2	1	Tuberculosis
27.9	9.4	18.5	1	2	2	2	2	2	Malignancy
26.4	10.9	15.5	1	3	2	2	3	4	Inconclusive
22.2	13.2	9	1.3	3	2	2	2	4	Atypical mesothelial proliferation
27.4	13.4	14	9	1	2	2	2	1	Tuberculosis
16.9	1.2	15.7	1.5	2	2	2	2	2	Carcinoma
5.6	2.6	3	1	3	2	2	2	4	Inconclusive
31.1	16.3	14.8	30	1	2	2	2	1	Tuberculosis
6.7	6	0.7	1	2	2	2	1	2	Adenocarcinoma
10.4	10.4	0	1	2	2	2	1	2	Adenocarcinoma
	5.2		1	1	2	2	2	2	Adenocarcinoma
11.3	8.1	3.2	8.23	1	2	2	2	1	Tuberculosis
	4.9		1	2	2	2	1	2	Adenocarcinoma
65.5	51	14.5	1	3	2	2	2	4	Inconclusive
9.1	4.4	4.7	1	3	2	2	2	4	Inconclusive
	17.8		28.6	1	2	1	2	1	Tuberculosis
152.7	36.6	116.1	1	2	2	2	2	3	Non-Hodgkin's lymphoma
38.3	36.7	1.6	30	1	2	1	2	1	Tuberculosis
	20.1		28.7	1	2	1	2	1	Tuberculosis
12.6	12.6	0	1	3	2	2	2	4	Inconclusive
75.2	49.4	25.8	1	3	2	2	2	4	Inconclusive
	19.4		30	1	2	1	2	1	Tuberculosis
8.6	3.1	5.5	1	2	2	2	2	2	Adenocarcinoma
132.3	23.3	109	30	3	2	1	3	1	Tuberculosis
	20.4		30	1	2	2	3	1	Tuberculosis
164.7	9.8	154.9	14.7	3	2	2	2	1	Tuberculosis
63	37.7	25.3	30	1	2	1	2	1	Tuberculosis
19.8	19.8	0	1	3	2	2	2	4	Churg Strauss syndrome

	9.9		3.66	1	2	2	2	1	Tuberculossis
118	36.7	81.3	30	1	2	2	2	1	Tuberculosis
8.3			20.3	3	2	2	3	4	Inconclusive
23.6	17.9	5.7	1	1	2	1	3	1	Tuberculosis
8.8	10.7		1	1	2	2	2	1	Tuberculosis
40.6	12.3	28.3	1	2	2	2	2	2	Adenocarcinoma
37.4	12.8	24.6	30	1	2	2	2	1	Tuberculosis
38.3	14.3	24	30	1	2	1	2	1	Tuberculosis
41.1	4	37.1	1	2	2	2	2	2	Adenocarcinoma
17.2	11.8	5.4	30	1	2	2	2	1	Tuberculosis
6.8	5.8	1	1	2	2	2	3	2	Adenocarcinoma
14.9	14.9	0	3.24	1	2	2	2	1	Tuberculosis
84.2	36.4	47.8	4.88	1	1	2	3	1	Tuberculosis
3.1	2	1.1	1	2	2	2	2	2	Adenocarcinoma
51.4	12.6	38.8	1	2	2	2	1	2	Poorly differentiated carcinoma
46.9	7.4	39.5	1	2	2	2	1	3	Adenocarcinoma
39.3	14.2	25.1	28.8	1	2	2	3	1	Tuberculosis
13.7	10	3.7	1	2	2	2	1	2	Adenocarcinoma
19.9	7.3	12.6	5.4	1	2	2	2	1	Tuberculosis
33.9	10.8	23.1	8.09	1	2	1	3	1	Tuberculosis
2.5	6.3		1	2	2	2	2	2	Adenocarcinoma
3.6	2	1.6	1	3	2	2	2	4	Probable mesothelioma
18.2	5.5	12.7	3	1	2	2	3	1	Tuberculosis
12.2	15.2		21.9	1	2	1	2	1	Tuberculosis
21.6	23.7		18.2	1	2	1	2	1	Tuberculosis
3.8	6.7		1	2	2	2	1	2	Adenocarcinoma
23.8	29.2		30	1	2	2	2	1	Tuberculosis
2	4.7		1	3	2	2	3	4	Inconclusive
5.7	2.7	3	1	3	2	2	3	4	Inconclusive
7.9	33.7		1	2	2	2	1	2	Adenocarcinoma
	22.7		20.3	3	2	2	2	4	Inconclusive
13.6	5	8.6	1	3	2	2	2	4	Inconclusive

11.3	11.3	0	1	2	2	2	1	2	Adenocarcinoma
92.3	25.8	66.5	30	1	2	2	2	1	Tuberculosis
22.9	4.6	18.3	4.6	3	2	2	3	4	Inconclusive
	4.1		3.6	3	2	2	2	3	Metastatic carcinoma+ pulm TB-TBLB
	4.9		1.64	1	2	2	2	1	Tuberculosis
	13.9		30	1	2	1	3	1	Tuberculosis
12.8	3.4	9.4	1	3	2	2	2	4	Inconclusive
13.1	8.3	4.8	1	3	2	2	2	4	Inconclusive
61.6	7.5	54.1	17.3	1	1	2	2	1	Tuberculosis
15.9	3.4	12.5	1	2	2	2	2	2	Adenocarcinoma
37.1	9.2	27.9	3.28	3	2	2	2	4	Inconclusive
13.2	5.7	7.5	1	3	2	2	2	4	Inconclusive
23.9	4.1	19.8	13	1	2	2	2	1	Tuberculosis
17.8	2.9	14.9	1	3	2	2	3	3	Non-Hodgkin's lymphoma
42.4	14.2	28.2	30	1	2	2	3	1	Tuberculosis+ HIV infection
	5.7		1.7	1	2	2	2	1	Tuberculosis
3.5	4.7		1	3	2	2	3	4	Inconclusive
6.6	4.6	2	1	3	2	2	2	4	Benign mesothelial proliferation
20.3	10.6	9.7	1.9	1	2	2	3	1	Tuberculosis
7.9	11.9		1	3	2	2	2	4	Inconclusive
56.9	9.6	47.3	12.6		1		3	1	Tuberculosis
17.4	7.2	10.2	1	3	2	2	3	4	Inconclusive
43.9	15.8	28.1	24.1	1	2	2	2	1	Tuberculosis
	17.6		30	1	2	2	2	1	Tuberculosis
9.5	3	6.5	1	3	2	2	2	4	Inconclusive
44.9	12.1	32.8	1	3	2	2	2	4	Inconclusive
83.2	33.8	49.4	30	1	2	2	2	1	Tuberculosis
32.1	13.4	18.7	1	1	2	2	2	1	Tuberculosis
11.8	6	5.8	1	2	2	2	2	2	Malignancy
39.7	14.5	25.2	1.01	1	2	2	2	1	Tuberculosis
65.1	12.4	52.7	20.6		2	2	3	4	Inconclusive
65.9	13.3	52.6	1	1	2	2	3	1	Tuberculosis

37.7	5.7	32	30	1	2	2	3	1	Tuberculosis
45.9	7	38.9	30	1				1	Tuberculosis
14.1	3.6	10.5	1	3	2	2	2	4	Aspergilloma?
12.1	15.7		1.79	2	2	2	2	2	Non-small cell carcinoma
67.5	9	58.5	30	1	2	2	2	1	Tuberculosis
11.2	3.1	8.1	1	1	2	2	2	1	Tuberculosis + small cell carcinoma
48.3	10.8	37.5	27.7	1	2	2	2	1	Tuberculosis
248.9	43.3	205.6	30	3	2	2	2	4	Tuberculosis
31	4.3	26.7	1	3	2	2	2	4	Inconclusive
78.7	13.6	65.1	30	1	2	2	3	1	Tuberculosis
13.7	2	11.7	1	3	2	2	2	1	Sjogren's syndrome+ Disseminated TB
122.2	31.8	90.4	1.29	3	2	2	2	4	Clostridium empyema
60.4	8.3	52.1	1	3	2	2	2	4	Left Pyogenic hydropneumothorax
10.5	2	8.5	1.4	2	2	2	2	3	Lymphoma
86.4	13.9	72.5	1	3	2	2	2	4	Right complicated parapneumonic effusion
14.5	2.5	12	1	3	2	2	2	4	Inconclusive
14.1	2.3	11.8	1	3	2	2	2	4	Eosinophilic effusion- Probable secondary to H.nana
9.9	2.1	7.8	1	3	2	2	2	2	Inconclusive
57.7	7.1	50.6	30	1	2	2	2	1	Tuberculosis
17.4	6.4	11	1	2	2	2	2	2	Adenocarcinoma
23.2	8.7	14.5	30	1	2	2	2	1	Tuberculosis
8.7	2.1	6.6	1	2	2	2	2	2	Adenocarcinoma
12.9	6.2	6.7	1.33	2	2	2	2	3	Lymphoma
12.2	9.5	2.7	6.68	3	2	2	2	2	Fibrous tumour
67.1	5.5	61.6	3.45	1	2	2	2	1	Tuberculosis
9.8	2	7.8	1	3	2	2	2	4	Inconclusive
30.8	6.6	24.2	1	3	2	2	2	4	Inconclusive